

**The roles and regulation of ubiquitin/ubiquitin-like protein  
conjugation pathways in responses to oxidative stress in  
*Schizosaccharomyces pombe*.**

**Marc David Flanagan, BSc (Hons) MRes**

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## **Declaration**

I certify that this thesis contains my own work, except where acknowledged, and that no part of this material has been previously submitted for a degree or any other qualification at this or any other university.

## Abstract

Ubiquitin and ubiquitin-like proteins (Ubls) are conjugated to proteins to regulate activity, stability, localisation or function. Ubiquitin/Ubl conjugation pathways are highly conserved in eukaryotes, and usually involve activating enzymes (E1s) and conjugating enzymes (E2s) specific for each ubiquitin/Ubl. Many studies have suggested that ubiquitin/Ubl conjugation pathways are important for oxidative stress resistance. However, there is much to learn regarding the roles of these pathways in oxidative stress responses. Additionally, limited studies in mammalian cells and yeast have indicated that certain E1s and E2s are redox-regulated, although how this relates to stress resistance is largely unclear, and these regulatory mechanisms have never been shown to be conserved in eukaryotes. Here, the roles and regulation of ubiquitin/Ubl conjugation pathways in responses to oxidative stress are investigated in *Schizosaccharomyces pombe*.

Firstly, while our previous research has shown that the budding yeast E2, Cdc34, is redox-regulated, it was unclear whether ubiquitination is redox-regulated in other organisms. Results presented here show that the fission yeast Cdc34 homologue, Ubc15, is redox-regulated, suggesting that redox regulation of specific ubiquitination events may be conserved. Furthermore, Ubc15 is important for resistance to oxidative stress in *S. pombe*. Secondly, the Ubl Urm1 is found to be important for resistance to a range of stress conditions in *S. pombe*, as in *S. cerevisiae*, thus demonstrating for the first time that urmylation has conserved roles in stress resistance in eukaryotes. Additionally, urmylation controls the activation of a conserved mitogen-activated protein kinase during exposure to H<sub>2</sub>O<sub>2</sub>. Finally, although autophagic Ubl conjugation is not important for oxidative stress responses in *S. pombe*, these investigations have identified an E2 with roles in oxidative stress responses and cell cycle control. Taken together, these findings advance the study of the roles of ubiquitin/Ubl conjugation pathways in responses to oxidative stress, and offer exciting prospects for future investigations.

This thesis is dedicated to my grandfather, David Ford, to whom I owe so much.



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## List of Abbreviations

AMP	Adenosine monophosphate
AMS	4-acetamido-4'-maleimidylstilbene-2, 2'-disulfonic acid
APC/C	Anaphase-promoting complex/cyclosome
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Cdk	Cyclin-dependent kinase
Cdk <sub>i</sub>	Cdk inhibitor
CRD	Cysteine-rich domain
CRL	Cullin-RING E3 ligase
DAPI	4'-6-diamidino-2-phenylindole
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DUB	Deubiquitinating enzyme
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EMM	Edinburgh minimal medium
ER	Endoplasmic reticulum
FITC	Fluorescein isothiocyanate
gDNA	Genomic DNA
GFP	Green fluorescent protein
Gpx	Glutathione peroxidase
Grx	Glutaredoxin
GSH	Reduced glutathione
GSSG	Oxidised glutathione

HECT	Homologous to E6-AP C-terminus
HU	Hydroxyurea
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
LB	Luria broth
LiAc	Lithium acetate
MAPK	Mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
Mcm	Methoxy-carbonyl-methyl
mETC	Mitochondrial electron transport chain
M <sub>r</sub>	Relative molecular mass
mtDNA	Mitochondrial deoxyribonucleic acid
NADPH	Nicotinamide adenine dihydrogen phosphate
NDSM	Negatively-charged amino acid-dependent SUMOylation motif
NEDD8	Neural precursor cell expressed, developmentally down-regulated 8
NEM	N-ethyl maleimide
NES	Nuclear export signal
NO	Nitric oxide
Nox	Non-phagocytic NADPH oxidase
O <sub>2</sub> <sup>-</sup>	Superoxide anion
OH <sup>·</sup>	Hydroxyl radical
ORF	Open reading frame
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PDSM	Phosphorylation-dependent SUMOylation motif
PE	Phosphatidylethanolamine
PEG	Polyethylene glycol

PIPES	Piperazine-N,N'-bis(2-ethanesulfonic acid)
PI3K	Phosphatidylinositol 3-kinase
PMSF	Phenylmethylsulfonyl fluoride
Prx	Peroxiredoxin
PTP	Protein tyrosine phosphatase
RING	Really interesting new gene
RLD	Rhodanese-like domain
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinases
SAMP	Small archaeal modifier protein
SAPK	Stress-activated protein kinase
SCF	Skp1-Cullin-F-box
SDS	Sodium dodecyl sulphate
SENP	Sentrin-specific protease
SOD	Superoxide dismutase
Srx	Sulfiredoxin
ssDNA	Salmon sperm DNA
SUMO	Small ubiquitin-related modifier
TBS	Tris-buffered saline
TBZ	Thiabendazole
TCA	Trichloroacetic acid
TCEP	Tris(2-carboxyethyl)phosphine
TE	Tris-ethylenediaminetetraacetic acid
tRNA	Transfer ribonucleic acid
Trx	Thioredoxin

Ubl	Ubiquitin-like protein
ULP	Ubl-specific protease
Urm1	Ubiquitin-related modifier 1
YE5S	Yeast extract medium with five amino acid supplements

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# Chapter 1. Introduction

## 1.1. Ubiquitin and Ubls

Post-translational modification can influence protein activity, function, interactions, localisation and stability, and is key for the regulation of many biological systems. Phosphorylation and acetylation are well-established protein modifications. However, phosphates and acetyl groups are very small modifiers. By conjugating polypeptides to proteins, it is possible to regulate many other properties of the substrate (including protein-protein interactions due to the multiple potential interaction surfaces of a polypeptide). Ubiquitin is a small protein with a  $\beta$ -grasp fold and a  $M_r$  of ~8.5kDa, and is a classic example of a polypeptide that can be conjugated to proteins. Ubiquitin is highly conserved in eukaryotes, but is not found in prokaryotes or archaea (Hochstrasser, 2000). Ubiquitin conjugation to proteins, or ‘ubiquitination’, often results in trafficking to the 26S proteasome, leading to degradation of the substrate protein. However, there are many non-proteolytic effects of ubiquitination, as will be discussed later. Ubls can also be conjugated to proteins, and largely resemble ubiquitin in their three-dimensional  $\beta$ -grasp structures. Many of these modifiers are also highly conserved in eukaryotes, and include SUMO, NEDD8 and Urm1 (**Table 1.1**). Although ubiquitin and Ubls share diglycine motifs at their C-termini, Ubls rarely show significant sequence homology with ubiquitin. However, ubiquitin and Ubl conjugation mechanisms are very similar, and result in the conjugation of these polypeptides to a wide range of substrates. Ubiquitin/Ubl conjugation pathways, and the effects of ubiquitin/Ubl conjugation on protein function, have been reviewed extensively (Herrmann et al., 2007; Hochstrasser, 2000, 2009; Kerscher et al., 2006; Welchman et al., 2005), and are discussed below.



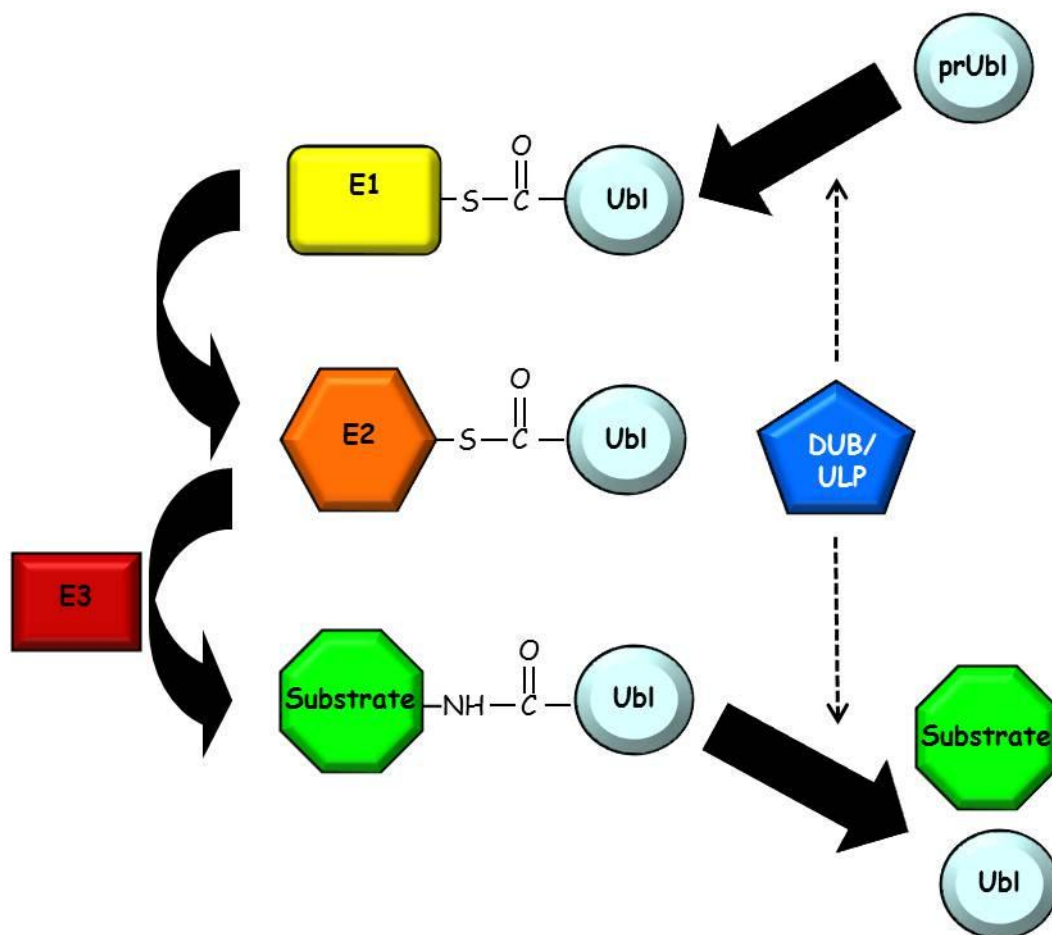
TABLE 1.1

### 1.1.1. Ubiquitin/Ubl conjugation pathways

#### *1.1.1.1. Ubiquitin/Ubl conjugation pathways – an overview*

Although there is some variation between ubiquitin/Ubl conjugation pathways in eukaryotes, a general schematic is shown in **Fig. 1.1**. An enzymatic cascade results in the conjugation of ubiquitin/Ubls to specific substrates. First, an activating enzyme (or 'E1') uses ATP to adenylate the ubiquitin/Ubl at the C-terminal. This allows an E1 catalytic cysteine residue to attack the high-energy carboxyl-AMP bond, forming a thioester bond between the cysteine and the C-terminal of the modifier, and releasing AMP. E1s often adenylate a second ubiquitin/Ubl molecule whilst binding the first molecule at the catalytic cysteine residue. A conjugating enzyme (or 'E2') then associates with the E1, allowing the transfer of ubiquitin/Ubl to the catalytic cysteine residue of the E2 via a transthioesterification mechanism. Finally, the ubiquitin/Ubl is conjugated to substrates, forming an isopeptide bond with target lysine residues. This final transfer step is often facilitated by ligase enzymes (or 'E3s'), which either form transient thioesters with the ubiquitin/Ubl before transferring the modifier to the substrate, or function as adaptor proteins to bring E2s and specific substrates together to allow conjugation directly from the E2. It is important to note that ubiquitin/Ubl conjugation is reversible, as certain cysteine proteases can hydrolyse isopeptide bonds between ubiquitin/Ubls and substrate lysines. These proteases are known as DUBs and ULPs. Additionally, these enzymes are often required to process ubiquitin and Ubls prior to conjugation, as ubiquitin and certain Ubls are synthesised in inactive forms with C-terminal extensions. Proteolytic cleavage by DUBs and ULPs exposes C-terminal diglycine motifs of ubiquitin and Ubls, allowing adenylation by E1s.

To allow the effective transfer of ubiquitin and Ubls to substrates, it is important that these modifiers are only transferred between enzymes in the direction shown in **Fig. 1.1**. Recent research has demonstrated the molecular basis for directional transfer of ubiquitin/Ubls between enzymes. The affinity of an E2 for an E1 is greatly increased by conformational changes within the E1 resulting from thioester formation, increasing the efficiency of ubiquitin/Ubl transfer to the E2 by transthioesterification



**Figure 1.1. A general schematic of ubiquitin/Ubl conjugation pathways.**

In the first step of ubiquitin/Ubl conjugation pathways, an activating enzyme (E1) adenylates the C-terminus of the ubiquitin/Ubl, then uses a catalytic cysteine residue to form a thioester bond to the modifier. A conjugating enzyme (E2) receives the ubiquitin/Ubl from the E1 via a transthioesterification reaction, again forming a thioester bond using a catalytic cysteine residue. The E2 usually plays a role in determining substrate specificity. A third enzyme (E3 ligase) is involved in transferring ubiquitin/Ubl to substrates, either by binding the modifier directly, or by co-recruiting the E2 and substrate to allow transfer from the E2 to the substrate. Ubiquitin/Ubls usually form isopeptide bonds to substrate lysine residues. DUB/ULP enzymes can hydrolyse these bonds, thus removing the ubiquitin/Ubl from the substrate. In many cases, DUBs and ULPs are also required to process the C-termini of ubiquitin/Ubl precursors (prUbls) to expose C-terminal diglycine motifs and allow activation by E1s.

(Schulman and Harper, 2009). Similarly, E3s appear to associate with E2-ubiquitin/Ubl complexes far more strongly than with free E2 (Kerscher et al., 2006). It has also been shown that there is an overlap between E1- and E3-binding surfaces within E2s (Eletr et al., 2005), implying that E1 and E3 enzymes cannot associate with E2s simultaneously. Again, this is important for effective directional transfer of ubiquitin and Ubls.

Although ubiquitin is not found in prokaryotes, bacterial sulphur transfer systems have been recognised as ancestors of eukaryotic ubiquitin/Ubl conjugation pathways (Hochstrasser, 2000, 2009). As part of key biosynthetic pathways, the sulphur carriers ThiS and MoeD transfer sulphur to precursors of thiamine and molybdenum cofactors, respectively (Duda et al., 2005; Lake et al., 2001; Lehmann et al., 2006; Rudolph et al., 2001; Xi et al., 2001). Like ubiquitin/Ubls, ThiS and MoeD have  $\beta$ -grasp folds and include diglycine motifs at their C-termini. Furthermore, the E1-like enzymes ThiF and MoeB catalyse the activation of ThiS and MoeD respectively. However, ThiF and MoeB appear to form acyl-disulfide bonds to ThiS and MoeD, which are resolved to facilitate thiocarboxylation of MoeD and ThiS at their C-termini. This is not the case for most eukaryotic E1s, which form thioester bonds to cognate ubiquitin/Ubls. However, a specific E1, Uba4, was recently shown to catalyse the thiocarboxylation of a Ubl, Urm1, and this modification is required for Urm1 conjugation to proteins (Van der Veen et al., 2011). Furthermore, Urm1 was initially identified in *Saccharomyces cerevisiae* by amino acid sequence homology shared with ThiS and MoeD (Furukawa et al., 2000). Urmylation will be described in more detail in Section 1.1.5. However, ubiquitin/Ubl conjugation appears to have evolved from bacterial sulphur transfer mechanisms, and urmylation may represent an evolutionary link between the two processes.

In addition, refinement of sequence analysis techniques has allowed the identification of other prokaryotic proteins with  $\beta$ -grasp folds, which may represent early forms of ubiquitin/Ubls (Iyer et al., 2006). Indeed, it has recently been shown that conjugation of a prokaryotic Ubl (Pup) to substrates promotes trafficking to the proteasome in *Mycobacterium tuberculosis* (Mukherjee and Orth, 2008; Pearce et al., 2008), much like ubiquitin in eukaryotes. There is also emerging evidence for an archaeal Ubl conjugation system, whereby  $\beta$ -grasp proteins with C-terminal diglycine motifs are attached to specific substrates (Humbard et al., 2010). These archaeal  $\beta$ -grasp proteins are known as SAMPs. Interestingly, proteasomal inhibition causes the accumulation of proteins modified with SAMP1 in *Haloferax volcanii* (Humbard et al., 2010), indicating

that modification with SAMP1 may target substrates to the proteasome, as seen for ubiquitin and Pup. Furthermore, SAMP conjugation is dependent on the E1-like protein UbaA, which shares sequence homology with Uba4, ThiF and MoeB (Miranda et al., 2011), and SAMPs may also function as sulphur carriers, like Urm1, ThiS and MoeD (Miranda et al., 2011). Clearly, protein modification with  $\beta$ -grasp polypeptides is an ancient regulatory mechanism in nature, and eukaryotic ubiquitin/Ubl conjugation pathways may have evolved from these prokaryotic and archaeal systems.

#### *1.1.1.2. E1 structure*

Many E1 structures have been determined, greatly enhancing our knowledge of these enzymes and how they function (Capili and Lima, 2007; Dye and Schulman, 2007; Schulman and Harper, 2009). Most E1s consist of an adenylation domain (highly similar to the structures of bacterial MoeB and ThiF), a catalytic domain housing the catalytic cysteine residue, and a C-terminal domain with a ubiquitin-like fold. Some E1s are monomeric, while others form heterodimers, the components of which resemble the N-terminal and C-terminal regions of monomeric E1s. These enzymes are highly selective for their cognate ubiquitin/Ubl (although some E1s can activate more than one Ubl, as outlined below). In general, selectivity appears to be conferred by (i) electrostatic attraction/repulsion between the E1 and different modifiers, (ii) non-covalent interactions between the E1 and the cognate ubiquitin/Ubl, and (iii) insertions unique to each E1, often near the catalytic cysteine (Schulman and Harper, 2009). Clearly, E1s also have to bind downstream E2s selectively. The ubiquitin-like domain of E1 enzymes has been shown to play an important role in E2 recruitment, although other domains may contribute to E2 binding and enhance specificity (Lois and Lima, 2005). Indeed, recent evidence suggests that the primary function of the ubiquitin-like domain is to obstruct the binding of non-cognate E2s, rather than to promote the binding of cognate E2s (Tokgoz et al., 2012). Structural characterisation has also shown that the three E1 domains are flexibly bound, allowing conformational changes required for transfer of ubiquitin/Ubl from the adenylation domain to the catalytic domain, and then from the catalytic domain to the downstream E2 (Capili and Lima, 2007; Dye and Schulman, 2007; Schulman and Harper, 2009). This flexibility may also be required for the formation of a transient microenvironment favouring deprotonation of the E1

catalytic cysteine residue, a key step during thioester formation (Lee and Schindelin, 2008).

#### *1.1.1.3. E2 structure*

As with E1s, E2s have been studied extensively, and there are many excellent reviews describing how E2 structure relates to function (Capili and Lima, 2007; Chen, 2007; Dye and Schulman, 2007; van Wijk and Timmers, 2010). Most E2s are small 15-20kDa proteins with strikingly similar core structures. These core structures include substrate/E3 binding sites, a catalytic site, and non-covalent ubiquitin/Ubl binding sites (Chen, 2007). Some bacterial proteins have also been found to resemble the E2 core structure (Iyer et al., 2006), demonstrating that this protein fold is highly conserved. Although there are relatively few conserved amino acids within the core structure, an HPN motif and some acidic residues are located near the catalytic cysteine residue in many E2s (Tolbert et al., 2005; Wu et al., 2003b). The asparagine residue of the HPN motif has been proposed to stabilise oxyanion intermediates formed from substrate lysines during isopeptide bond formation (Wu et al., 2003b). Many E2s also have N- and C-terminal extensions. An example of this is the C-terminal ‘tail’ of the ubiquitin-specific E2 Cdc34, which is rich in acidic residues. There is considerable variation in the sizes of these extensions, meaning that the molecular weight of E2s can vary enormously (van Wijk and Timmers, 2010). E2 often have overlapping E1- and E3-binding sites. This has been shown for many different E2s functioning in different ubiquitin/Ubl conjugation systems (Huang et al., 2005; Huang et al., 1999; Reverter and Lima, 2005), and explains the finding that E2s cannot bind to E1s and E3s simultaneously (Eletr et al., 2005). The lack of a significant conformational change within E2s upon binding an E1 or an E3 may reflect the fact that E1- and E3-binding sites overlap (Huang et al., 2005; Huang et al., 1999; Zheng et al., 2000). However, as mentioned above, the formation of a thioester between an E2 and ubiquitin/Ubl can increase the affinity of an E3 for the E2.

## 1.1.2. Ubiquitination

### 1.1.2.1. Ubiquitin conjugation

Ubiquitin can be conjugated to more substrate proteins than any Ubl. The basis for this wide range of substrates lies in the number of enzymes that can function in ubiquitination. Ubiquitination is largely mediated by a single E1 (Uba1), although recent work has identified Uba6 as a bifunctional E1 that activates ubiquitin and a Ubl, FAT10, in higher eukaryotes (**Table 1.1**) (Chiu et al., 2007; Groettrup et al., 2008). Uba1 can transfer ubiquitin to many downstream E2s, and appears to bind these enzymes with similar affinities (Tokgoz et al., 2012). Uba1-E2 interactions are facilitated by the ubiquitin-like domain of Uba1 and Uba1-interacting motifs located near the N-termini of E2s, which include three conserved basic residues (Tokgoz et al., 2012). Substrates can either be modified by a single ubiquitin molecule (monoubiquitination) or by ubiquitin chains linked by lysine residues within the ubiquitin polypeptide (polyubiquitination). Polyubiquitination can occur through many different mechanisms, including rapid association and dissociation of E2s and E3s (Kleiger et al., 2009), and the sequential action of different E3s (Harreman et al., 2009).

Ubiquitin-specific E3 ligases fall into two major groups. HECT-type E3s use a catalytic cysteine residue to form a thioester bond to ubiquitin, and conjugate ubiquitin to downstream substrates. In contrast, RING-type E3s do not directly bind ubiquitin, instead functioning as adaptors to bring ubiquitin-loaded E2 enzymes and specific substrates together, allowing conjugation by the E2 (Petroski and Deshaies, 2005). The modular nature of RING-type E3s results in significant structural variation, allowing these enzymes to bind many different substrates that can be modified with ubiquitin. The majority of these E3s are cullin-RING ligases, which are centred on scaffold proteins called cullins. These proteins include variable N-terminal domains that bind specific adaptors which can then recruit substrate-binding proteins. For example, SCF E3s include the scaffold protein cullin-1 and the adaptor protein Skp1, which recruits substrate-binding proteins containing F-box motifs (Petroski and Deshaies, 2005). Cullins also have C-terminal domains that bind RING subunits important for the

recruitment of specific E2s. The recruitment of E2s is also enhanced by cullin neddylation (see Section 1.1.3).

#### *1.1.2.2. Targets and functions of ubiquitination*

Ubiquitination of proteins often results in proteasomal degradation (Glickman and Ciechanover, 2002). However, there are also many non-proteolytic functions of ubiquitination (Chen and Sun, 2009; Herrmann et al., 2007; Welchman et al., 2005). Strong correlations have been observed between the nature of protein ubiquitination and the resulting effects on the target protein. For example, lysine 48 (K48)-linked polyubiquitin chains usually target substrates to the 26S proteasome, whilst other modifications such as monoubiquitination and lysine 63 (K63)-linked polyubiquitin chains are non-proteolytic (Ikeda and Dikic, 2008). There is emerging evidence that E2s do not only promote ubiquitination of a specific substrate, but can also facilitate the conjugation of polyubiquitin chains linked by specific lysines, and thus determine the functional consequences of protein ubiquitination (David et al., 2010; Rodrigo-Brenni et al., 2010). The different consequences of ubiquitination can be explained by the proteins recruited by different ubiquitin chains. Many proteins include ubiquitin-binding domains, which often interact with a hydrophobic patch on the surface of ubiquitin (Chen and Sun, 2009). However, these interactions are often weak, and additional contacts must be made between the protein and the ubiquitin chain to allow binding. Thus, it follows that proteasomal subunits can make additional contacts to K48-linked polyubiquitin chains, but not to K63-linked chains, for example, as these chains have a very different structure. In contrast, other proteins will be able to bind K63-linked chains more strongly, resulting in non-proteolytic effects of ubiquitination, centred around the recruitment of ubiquitin-binding proteins (Chen and Sun, 2009).

Proteasomal degradation of proteins following polyubiquitination is critical to the regulation of many processes, including the cell cycle. Polyubiquitination triggers the degradation of key regulatory proteins such as cyclins, Cdkis and the key mitotic protein securin, to control the timing and duration of events in the cell cycle (Glötzter et al., 1991; King et al., 1996). For example, the E2, Cdc34, facilitates the ubiquitination



and degradation of the Sic1 protein in *S. cerevisiae* (Schwob et al., 1994). This is required for entry to S phase, as Sic1 inhibits the activity of a key Cdk, Cdc28, during G1 phase (Mendenhall, 1993). Polyubiquitination also controls the temporal degradation of key kinases, such as Wee1 (Michael and Newport, 1998), and phosphatases, such as Cdc25 (Nefsky and Beach, 1996), which control Cdk activity to regulate the onset of mitosis. The susceptibility of individual proteins to ubiquitination is regulated in a variety of ways (Kornitzer and Ciechanover, 2000). For example, Sic1 is phosphorylated by G1 Cdk such as Cdc28 (Feldman et al., 1997; Skowyra et al., 1997; Verma et al., 1997) and Pho85 (Nishizawa et al., 1998), triggering its ubiquitination. Furthermore, RING-type E3s such as SCFs and APC/C interact with activators and substrate recognition proteins at different points during the cell cycle, leading to degradation of specific substrates at appropriate times (Guardavaccaro and Pagano, 2004; Nakayama and Nakayama, 2006). As well as the cell cycle, ubiquitin-mediated proteolysis is implicated in bulk protein turnover, and many other processes including signal transduction, transcription and cell death (Hershko and Ciechanover, 1998).

Additionally, non-proteolytic ubiquitination is involved in many processes, including DNA repair. For example, PCNA is a nuclear protein that functions as a sliding clamp during DNA replication, and is modified by monoubiquitination by the E2, Rad6, and the E3, Rad18, in response to UV exposure or DNA damage (Hoege et al., 2002). Monoubiquitination allows PCNA to recruit specialised ‘translesion synthesis’ DNA polymerases that can bypass areas of DNA damage, allowing replication to continue (Haracska et al., 2004). Protein localisation can also be regulated by ubiquitination. For example, the transcription factor p53 is exported from the nucleus following ubiquitination by Mdm2 (Li et al., 2003a), or by MSL2 (Kruse and Gu, 2009). In addition, monoubiquitination of receptors has been shown to promote endocytosis by facilitating the recruitment of endocytic adaptor proteins containing ubiquitin-binding domains within their structures (Haglund et al., 2003). Non-proteolytic ubiquitination of histones is also important for epigenetic regulation of gene expression. For example, Rad6 catalyses the ubiquitination of histone H2B following DNA damage, which promotes the subsequent methylation of histone H3 and leads to repression of transcription (Sun and Allis, 2002).

#### *1.1.2.3. Deubiquitination*

Deubiquitination is also an important process in eukaryotic cells. The human genome encodes ~100 DUBs, each catalysing the deubiquitination of specific proteins. The majority of DUBs are cysteine proteases that use a negatively-charged cleft to bind near the C-terminal of ubiquitin (Song and Rape, 2008). The regulation of DUB activity is now associated with the control of specific processes in the cell, including the cell cycle. For example, Usp1 catalyses the deubiquitination of PCNA under homeostatic conditions (Huang et al., 2006). However, exposure of cells to UV light triggers the autocleavage of Usp1, thus increasing PCNA monoubiquitination during S phase, which is important for the recruitment of 'translesion synthesis' DNA polymerases, as outlined above (Haracska et al., 2004; Huang et al., 2006). A second DUB, Usp44, catalyses the deubiquitination of Cdc20, a protein with a role in regulating anaphase (Song and Rape, 2008; Stegmeier et al., 2007). Usp44 is inhibited by phosphorylation during M phase, resulting in increased non-proteolytic ubiquitination of Cdc20, activation of APC/C, and the onset of anaphase (Song and Rape, 2008; Stegmeier et al., 2007). As DUBs regulate the activity and stability of specific proteins functioning in the cell cycle, they may eventually be targeted by therapeutics during treatment of diseases such as cancer (Song and Rape, 2008).

#### *1.1.2.4. Ubiquitination and disease*

The gene encoding ubiquitin is essential in eukaryotes. Furthermore, due to the key role played by ubiquitin-mediated proteolysis in the control of cell cycle progression, it is essential that ubiquitination is controlled appropriately to prevent cell cycle dysfunction, which can result in diseases such as cancer (Guardavaccaro and Pagano, 2004; Nakayama and Nakayama, 2006). Non-proteolytic ubiquitination is also important for cancer prevention. Additionally, neurodegenerative disorders such as Alzheimer's disease and Huntington's disease are associated with a build-up of protein aggregates. Ubiquitination of these aggregates is important for their removal, and helps prevent disease. For example, Huntington's disease is caused by the accumulation of a mutant Huntingtin protein, which causes neuronal degeneration (Landles and Bates, 2004).

Ubiquitination of this mutant protein triggers its degradation, thus preventing disease pathogenesis (Bjorkoy et al., 2005). Non-proteolytic ubiquitination is also important for cancer prevention. As described above, the non-proteolytic ubiquitination of Cdc20 is important for the timing of anaphase, and any disruption could result in heritable genetic damage that is characteristic of cancer cells (Nakayama and Nakayama, 2006).

Although ubiquitination is clearly important for the prevention of disease, the ubiquitination of specific proteins has also been implicated in disease. Indeed, proteasomal inhibitors have been used to treat inflammatory diseases, as the activity of pro-inflammatory transcription factors such as NF $\kappa$ B is dependent on the ubiquitination and degradation of inhibitors such as I $\kappa$ B (Bedford et al., 2011). However, this is complicated by the role of ubiquitin-mediated proteolysis in key cellular processes such as the cell cycle. Thus, the design of inhibitors of specific E2s and E3s is an interesting prospect, as these inhibitors could prevent the ubiquitination of specific proteins, but allow overall ubiquitination to continue unaffected (Bedford et al., 2011).

### **1.1.3. SUMOylation**

#### *1.1.3.1. SUMO conjugation*

SUMO has a very similar overall structure to ubiquitin, but has a different distribution of amino acid residues on its surface, and contains an N-terminal extension not found in ubiquitin (Bayer et al., 1998). Like ubiquitin, SUMO is present as a single isoform in lower eukaryotes, but unlike ubiquitin, multiple SUMO isoforms are expressed in mammalian cells. The highly-conserved SUMO conjugation (or ‘SUMOylation’) pathway attaches all SUMO isoforms to substrates. A single E1 activates SUMO, as with ubiquitin. However, unlike monomeric Uba1, the SUMO E1 is heterodimeric (Uba2/Aos1; henceforth referred to as Uba2) (Johnson et al., 1997). A single E2, Ubc9, has been shown to function in SUMOylation (Desterro et al., 1997; Johnson and Blobel, 1997; Lee et al., 1998a), perhaps explaining why SUMO is conjugated to fewer proteins

than ubiquitin. In contrast to ubiquitination, SUMOylation is often targeted to a lysine residue within a specific consensus motif,  $\Psi$ KXD/E (where  $\Psi$  is hydrophobic and X can be any amino acid) (Rodriguez et al., 2001). Furthermore, some SUMO substrates include NDSMs or PDSMs, comprising the  $\Psi$ KXD/E motif and downstream acidic residues or phosphorylated serine/threonine residues, respectively (Anckar and Sistonen, 2007). These negatively-charged species appear to enhance SUMOylation via an interaction with a positively-charged, basic patch on the surface of Ubc9 (Mohideen et al., 2009). The fact that consensus motifs can direct SUMOylation may reflect the fact that, unlike for ubiquitination, only one E2 is involved in SUMOylation, making interactions between Ubc9 and the surface of the substrate more important for targeting SUMO to specific proteins. Many SUMO E3s have been identified, most notably PIAS proteins, and ULP/SENPs function as deSUMOylase enzymes (Gareau and Lima, 2010; Geiss-Friedlander and Melchior, 2007; Hay, 2005).

#### *1.1.3.2. Targets and functions of SUMOylation*

In contrast to ubiquitination, SUMOylation does not target proteins to the proteasome. In many cases, SUMO mediates protein-protein interactions. For example, the first SUMO substrate to be identified was RanGAP1, which localises to the nuclear pore complex via a SUMO-dependent interaction with the nucleoporin RanBP2 (Mahajan et al., 1997; Matunis et al., 1996). The study of protein-protein interactions has led to the identification of SUMO-interacting motifs, usually consisting of stretches of hydrophobic amino acids flanked by acidic residues, which may interact with regions on the surface of SUMO that are not found in other UbIs (Gareau and Lima, 2010; Song et al., 2004).

Analyses of SUMOylated proteins in *S. cerevisiae* have shown that many SUMO substrates are nuclear proteins (Wohlschlegel et al., 2004; Zhou et al., 2004). For example, transcription factors such as c-Fos and c-Jun are SUMOylated to repress their activity, possibly by recruitment of co-repressor proteins (Bossis et al., 2005; Hay, 2005). SUMO can also repress transcription more directly, as SUMOylation of the heterochromatin proteins Swi6 and Clr4 promotes their activity, leading to histone H3

methylation by Clr4 and subsequent Swi6 binding to the methylated histone, which are important steps in heterochromatin formation (Shin et al., 2005). Another well-known substrate is the DNA base excision repair enzyme thymine-DNA glycosylase, which is involved in the repair of thymine/uracil mismatches. SUMOylation of thymine-DNA glycosylase decreases its affinity for DNA, allowing the enzyme to dissociate from DNA following the generation of an abasic site (Hardeland et al., 2002). As well as nuclear proteins, some cytoplasmic SUMO substrates have been identified, including RanGAP1 (as discussed above) and I $\kappa$ B $\alpha$ . However, in many cases, the functional consequences of SUMOylation remain unknown.

Ubiquitination and SUMOylation are clearly distinct pathways. However, there are many examples of cross-talk between the two pathways. For example, the ubiquitin-specific E2, E2-25K, is inhibited by SUMOylation (Pichler et al., 2005), and some ubiquitin-specific E3s are recruited to substrates previously modified by SUMO (Heideker et al., 2009). Ubiquitin and SUMO can also target the same lysine residues. Indeed, it has been proposed that one of the key functions of SUMOylation is to block other post-translational modifications of lysine residues, including ubiquitination (Zhao, 2007). For example, SUMOylation can block ubiquitination of PCNA during S phase, thus preventing the regulation of PCNA activity by non-proteolytic ubiquitination, as outlined previously (Hoege et al., 2002). Furthermore, I $\kappa$ B $\alpha$  is modified by SUMO to block ubiquitination, thus increasing the stability of I $\kappa$ B $\alpha$  and inhibiting NF $\kappa$ B activity (Desterro et al., 1998; Zhao, 2007). However, phosphorylation of I $\kappa$ B $\alpha$  prevents SUMOylation and promotes ubiquitination (Desterro et al., 1998), again demonstrating that ubiquitin/Ubl conjugation can be regulated by other post-translational modifications of a particular substrate.

#### *1.1.3.3. SUMOylation and disease*

The genes encoding SUMO, Uba2, Aos1 and Ubc9 are all essential in budding yeast, *S. cerevisiae* (Dasso, 2008), and inhibition of SUMOylation causes cell cycle arrest (Dieckhoff et al., 2004). SUMO is not essential in the fission yeast, *Schizosaccharomyces pombe*, but a dysfunctional SUMOylation pathway leads to slow

growth and numerous chromosomal defects (Dasso, 2008). Indeed, even before SUMOylation was characterised, *S. pombe*  $\Delta uba2$  and  $\Delta ubc9$  cells were found to be highly-sensitive to DNA damage caused by HU or UV exposure, and were defective in chromosome segregation during mitosis (al-Khodairy et al., 1995; Shayeghi et al., 1997). These results show that SUMO has important roles in DNA replication and repair and in mitosis, and it is vital that the SUMOylation pathway remains functional to prevent disease. Indeed, dysfunctional SUMOylation has been linked to human diseases including cancer (Zhao, 2007).

Like ubiquitination, SUMOylation is involved in the control of cell cycle progression. In many cases, the coordination of ubiquitination and SUMOylation appears to be important for the control of key events during the cell cycle. For example, SUMOylation of the ubiquitin-specific E3, APC/C, is important for its activation and for the timing of chromosome separation during anaphase (Dieckhoff et al., 2004; Gutierrez and Ronai, 2006). SUMOylation may also influence the development of neurodegenerative diseases such as Huntington's disease, as SUMOylation of the mutant Huntingtin protein increases the toxicity of the protein (Steffan et al., 2004). Finally, the bacterium *Listeria monocytogenes* blocks SUMOylation during infection by causing the degradation of Ubc9, an event required for efficient infection (Ribet et al., 2010). This suggests that SUMOylation has roles in defence against pathogens.

#### **1.1.4. Neddylation**

##### *1.1.4.1. NEDD8 conjugation*

Similarly to SUMOylation, the highly-conserved NEDD8 conjugation (or 'neddylation') pathway consists of a single E1, the Uba3-APPBP1 heterodimer (henceforth referred to as Uba3) and an E2, Ubc12 (Gong and Yeh, 1999; Liakopoulos et al., 1998; Osaka et al., 1998). Uba3 activates NEDD8, with specificity conferred by hydrophobic interactions between Uba3 and the Ala72 residue of NEDD8 (Walden et

al., 2003). In contrast, ubiquitin and SUMO have polar residues at position 72. However, recent evidence suggests that Uba1 can also activate NEDD8 in the absence of sufficient competition from ubiquitin (Leidecker et al., 2012). Ubc12 specifically interacts with NEDD8-loaded Uba3, due to contacts formed between loop regions of Uba3 and an N-terminal extension specific to Ubc12 (Huang et al., 2004). Unlike ubiquitin, NEDD8 does not form polymeric chains after conjugation to substrates (Girdwood et al., 2011), although activation by Uba1 may result in the formation of polymeric chains involving both ubiquitin and NEDD8 (Leidecker et al., 2012). Removal of NEDD8 from substrates is often mediated by the COP9 signalosome (Lyapina et al., 2001). However, other NEDD8-specific proteases have been identified, including the mammalian enzyme NEDP1, which is also able to process newly-synthesised NEDD8 into the mature form that can be activated by Uba3 (Wu et al., 2003a).

#### *1.1.4.2. Targets and functions of neddylation*

The cullin subunits of CRLs are modified by neddylation near their C-termini (Osaka et al., 1998), and are recognised as the major target of neddylation in eukaryotic cells (Bosu and Kipreos, 2008; Pan et al., 2004; Petroski and Deshaies, 2005). Dcn1 has been identified as a conserved NEDD8-specific E3 ligase promoting cullin neddylation, and functions as an adaptor protein to co-recruit Ubc12 and cullins (Kurz et al., 2008). NEDD8 may 'activate' CRLs by inducing conformational changes within their structure (Rabut and Peter, 2008). Indeed, neddylation of CRLs facilitates the recruitment of ubiquitin-loaded E2s (Kawakami et al., 2001). In addition, NEDD8 may bridge the gap between the ubiquitin-loaded E2 and the substrate co-recruited by a CRL to promote ubiquitination of the substrate (Saha and Deshaies, 2008). It is also clear that cycles of neddylation and deneddylation are required for optimal CRL activity, as prolonged neddylation of cullins increases the likelihood of autoubiquitination and degradation (Bosu and Kipreos, 2008). The link between neddylation and CRL activity implies that NEDD8 can control the ubiquitination of many proteins indirectly.

The importance of the neddylation pathway is clear, as the gene encoding NEDD8 is essential in lower eukaryotes, such as *S. pombe*, and in higher eukaryotes, such as mice (although, interestingly, budding yeast cells lacking a functional neddylation pathway remain viable) (Liakopoulos et al., 1998; Osaka et al., 2000; Tateishi et al., 2001). CRLs promote the ubiquitination and degradation of numerous cell cycle regulators, transcription factors and signalling molecules (Bosch and Kipreos, 2008; Petroski and Deshaies, 2005). Thus, CRL substrates accumulate when neddylation is inhibited in mammalian cells, leading to morphological changes associated with aberrant cell cycle progression, such as cell enlargement (Leck et al., 2010). As CRL activity is important for cell viability, and may become dysfunctional during tumourigenesis, neddylation has been identified as a possible target for cancer therapies (Guardavaccaro and Pagano, 2004; Soucy et al., 2009). Indeed, an inhibitor of Uba3 has been effective in targeting leukaemia cell lines (Swords et al., 2010). Furthermore, inhibition of neddylation caused leukaemia cell death partly by increasing ROS generation (Swords et al., 2010), implying a role for neddylation in oxidative stress resistance.

Although cullins are major neddylation substrates, additional targets have also been identified (Rabut and Peter, 2008; Xirodimas, 2008). A significant target is p53, which is inhibited by neddylation (Xirodimas et al., 2004). The E3 ligase required for p53 neddylation was identified as Mdm2 (Xirodimas et al., 2004), which also promotes mono- and polyubiquitination of p53 (Li et al., 2003a). This demonstrates that E3s can be bifunctional, conjugating ubiquitin and NEDD8 (two very similar proteins) to substrates (Xirodimas, 2008).

### **1.1.5. Urmylation**

#### *1.1.5.1. Urm1 conjugation*

Urm1 was originally identified due to similarities with the bacterial sulphur carriers MoeD and ThiS, as it shows little sequence homology to ubiquitin or other Ubbs

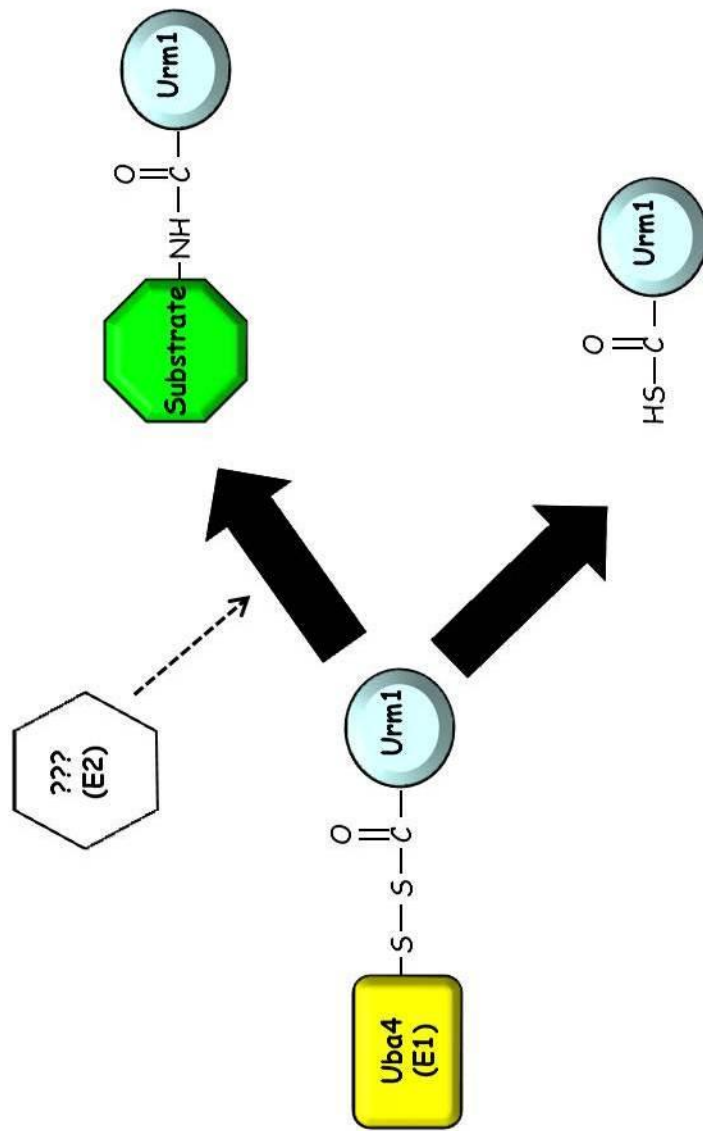


(Furukawa et al., 2000). Clear similarities between Urm1 and MoeD/ThiS can also be seen at the structural level (Xu et al., 2006). Furthermore, the Urm1-specific E1, Uba4, closely resembles the bacterial sulphurtransferases MoeB and ThiF, although Uba4 is similar to other E1s in some areas, including the ATP-binding site (Furukawa et al., 2000). Analytical ultracentrifugation analysis suggests that Uba4 forms homodimers (Schmitz et al., 2008), although the importance of homodimerisation in enzyme function is unclear. The activation of Urm1 by Uba4 is different to the activation of ubiquitin or other UbIs, as Uba4 also transfers sulphur to the C-terminus of Urm1 by thiocarboxylation (Krepinsky and Leimkuhler, 2007; Schmitz et al., 2008). This mechanism supports the idea that Urm1 links prokaryotic sulphur transfer and eukaryotic ubiquitin/Ubl conjugation, and may represent an evolutionary link between these two systems (Schmitz et al., 2008). Interestingly, no Urm1-specific E2 and E3 enzymes have been identified (**Fig. 1.2**). However, Urm1 is conjugated to lysine residues of specific substrates (Goehring et al., 2003a; Van der Veen et al., 2011), thus demonstrating that Urm1 can function in both sulphur transfer and protein modification (Pedrioli et al., 2008; Wang et al., 2011).

#### 1.1.5.2. Targets and functions of urmylation

*S. cerevisiae*  $\Delta$ urm1 and  $\Delta$ uba4 mutants display increased sensitivity to heat, rapamycin and oxidative stress (Furukawa et al., 2000; Goehring et al., 2003a), and are defective in pseudohyphal growth and invasion during nutrient limitation (Goehring et al., 2003b). Clearly, urmylation is required to protect cells against these stresses. It is possible that the roles of Urm1 in protein modification and sulphur transfer are both important for stress resistance.

The first urmylation substrate to be identified was the *S. cerevisiae* antioxidant enzyme Ahp1 (Goehring et al., 2003a). Similar to  $\Delta$ urm1 and  $\Delta$ uba4,  $\Delta$ ahp1 cells are sensitive to oxidative stress. Furthermore, urmylation of Ahp1 was increased when cells were exposed to oxidative stress agents such as diamide, but decreased in response to other agents, such as *t*-BOOH (Goehring et al., 2003a). Thus, urmylation of Ahp1 might be predicted to regulate the activity of the enzyme in response to different oxidative stress



**Figure 1.2. The urmylation pathway differs from canonical ubiquitin/Ubl conjugation pathways.**

Urmylation has been described as a bifunctional pathway, as it can function in both sulphur transfer and protein modification. Urm1 is conjugated to specific proteins, and can act as a sulphur carrier due to thiocarboxylation of the C-terminus by the E1 enzyme, Uba4, which may form a disulfide bond to Urm1 rather than a thioester bond. No Urm1-specific E2 enzyme(s) have yet been identified. Indeed, it is possible that Uba4 confers some substrate selectivity to the pathway, in contrast to E1s in other ubiquitin/Ubl conjugation pathways. As with other ubiquitin/Ubl conjugation pathways, Urm1 appears to be conjugated to substrate lysines via isopeptide bonds.

agents. However, it remains unclear how urmylation of Ahp1 affects the function of the enzyme. Furthermore, *Δurm1* and *Δuba4* cells are more sensitive to diamide and less sensitive to *t*-BOOH than *Δahp1* cells (Goehring et al., 2003a), suggesting that the stress sensitivities resulting from dysfunctional urmylation may not be due to changes in Ahp1 activity. Several new urmylation substrates have recently been identified in mammalian cells, and urmylation is increased when mammalian cells are treated with diamide, as in *S. cerevisiae* (Van der Veen et al., 2011). However, no conserved Urm1 substrates have been identified, and no functional consequence of protein urmylation has been determined. To shed more light on the functions of urmylation, it will be important to show the effects of urmylation on protein activity.

Additionally, Urm1 functions as a sulphur carrier protein as part of a tRNA modification pathway, which has largely been characterised in yeast (Bjork et al., 2007; Dewez et al., 2008; Huang et al., 2008; Leidel et al., 2009; Nakai et al., 2008; Noma et al., 2009). Firstly, the mitochondrial cysteine desulphurase Nfs1 provides sulphur atoms to allow the thiocarboxylation of Urm1 by Uba4 (a second protein, Tum1, may also promote thiocarboxylation by shuttling sulphur from the mitochondria into the cytoplasm). Urm1 then functions in conjunction with the ATPases Ncs2 and Ncs6 to promote 2-thiolation of the wobble uridines of tRNA<sup>Lys</sup><sub>UUU</sub>, tRNA<sup>Glu</sup><sub>UUC</sub> and tRNA<sup>Gln</sup><sub>UUG</sub>. These wobble uridines are also modified with mcm groups by the Elongator complex (Svejstrup, 2007), which is required for their subsequent thiolation (Leidel et al., 2009; Noma et al., 2009). Modification of tRNA<sup>Lys</sup><sub>UUU</sub>, tRNA<sup>Glu</sup><sub>UUC</sub> and tRNA<sup>Gln</sup><sub>UUG</sub> is important for maintaining translational fidelity, as it is required for interactions with the ribosome and to reduce the flexibility of anticodons to promote translational fidelity (Ashraf et al., 1999; Bjork et al., 2007; Yarian et al., 2002).

The role of Urm1 as a sulphur carrier may also be important for stress resistance. For example, it has been reported that tRNA overexpression can rescue the increased sensitivity of *S. cerevisiae* *Δurm1* cells to diamide and rapamycin (Leidel et al., 2009), indicating that *Δurm1* cells display increased stress sensitivity due to loss of tRNA modification, not due to the loss of protein urmylation. However, strikingly, these investigations did not show whether tRNA overexpression rescued the temperature sensitivity of *Δurm1* (Leidel et al., 2009). It is possible that the temperature sensitivity of *Δurm1* was not fully rescued by tRNA overexpression, and that *Δurm1* cells are temperature sensitive due to loss of both sulphur transfer and protein modification.

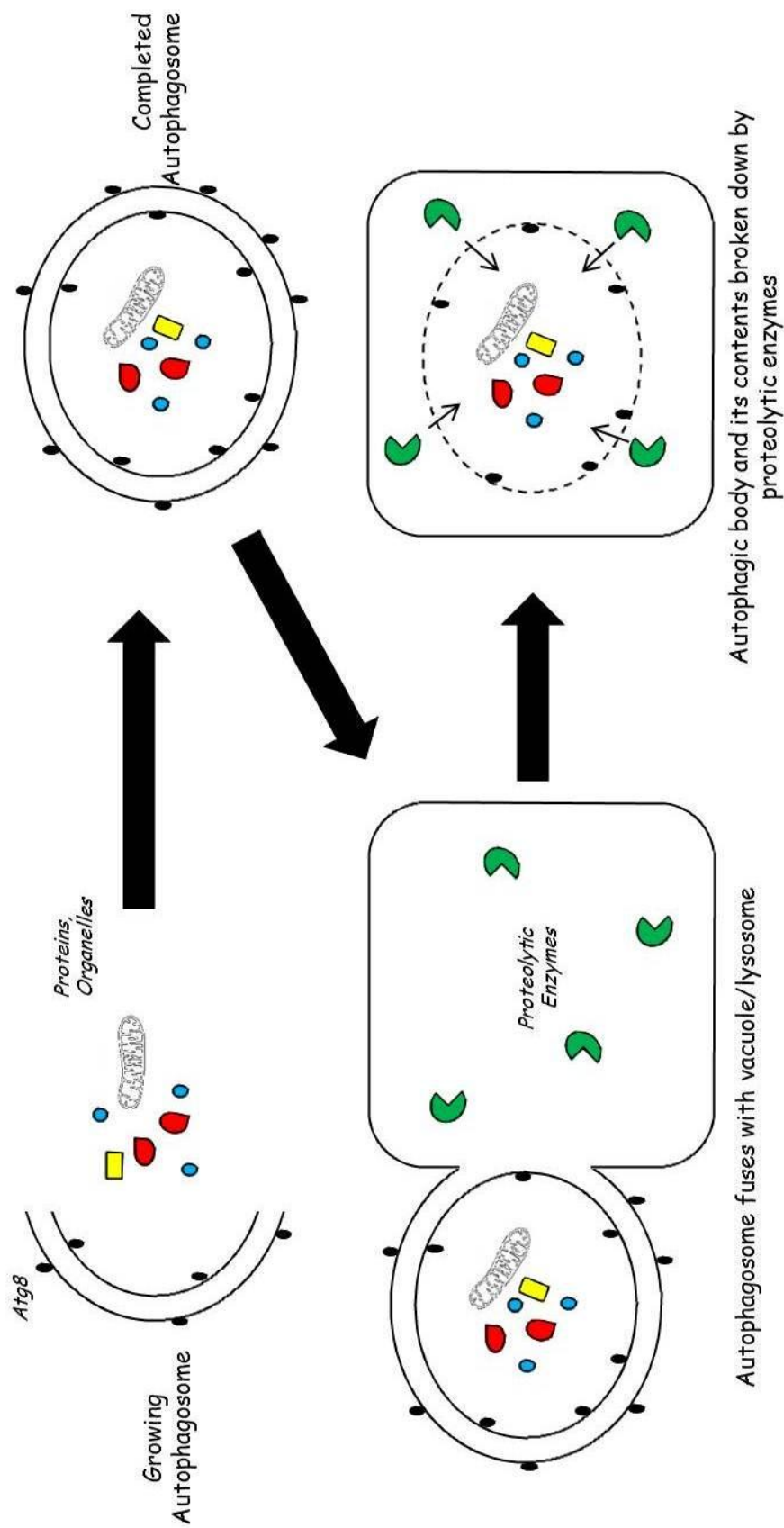
Additionally, the rapamycin sensitivity of *S. cerevisiae* *Δurml* cells can also be rescued by loss of Gln3, a transcription factor whose nuclear localisation is indirectly controlled by urmylation (Rubio-Teixeira, 2007), or by the expression of a rapamycin-resistant TOR (Goehring et al., 2003b), adding further layers of complexity to the role of urmylation in stress resistance.

### **1.1.6. Autophagic Ubl conjugation**

#### *1.1.6.1. Autophagy*

As outlined above, ubiquitination plays a significant role in protein degradation in eukaryotes. However, macroautophagy – a process of cellular self-digestion, hereafter referred to as ‘autophagy’ – is also involved in the turnover of proteins and organelles in eukaryotes (Reggiori and Klionsky, 2002). During autophagy, a double-membrane vesicle – known as the autophagosome – is formed around cytoplasmic components, before fusing with the lysosome to trigger the proteolytic degradation of its contents (**Fig. 1.3**). Autophagy is very similar in lower eukaryotes, but autophagosomes fuse with vacuoles in yeast, rather than lysosomes (Kiel, 2010). Although autophagy allows the basal turnover of proteins and organelles under homeostatic conditions, the process can be dramatically induced in response to certain stimuli (see Section 1.1.6.4). For example, autophagic activity is increased under starvation conditions to provide a source of amino acids. This allows protein synthesis to continue in order to maintain viability (Onodera and Ohsumi, 2005).

Although autophagy generally breaks down cellular components in a non-selective manner, there are many instances of selective autophagy. For example, in *S. cerevisiae*, the acetaldehyde dehydrogenase enzyme Ald6 is preferentially degraded during starvation (although how this is achieved and how it may benefit the cell remains unclear) (Onodera and Ohsumi, 2004). Additionally, if mammalian cells are treated with caspase inhibitors to prevent apoptosis, the antioxidant enzyme catalase is targeted for



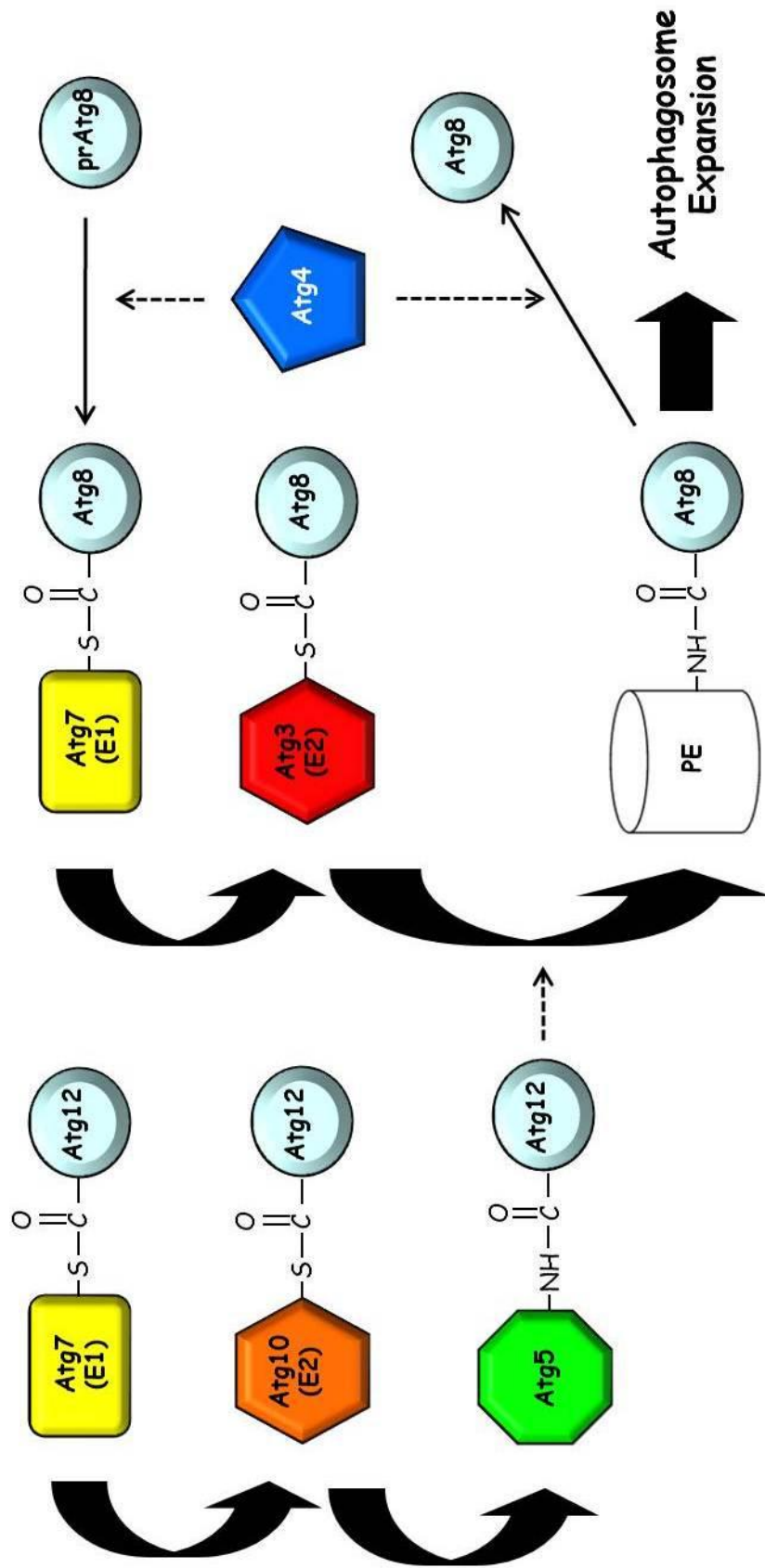
**Figure 1.3. Autophagy as a process of cellular degradation.**

Autophagy is a process by which cytosolic components are trafficked to the vacuole/lysosome and degraded. Autophagy occurs at a basal level in eukaryotes, but can be upregulated in response to various stimuli. A double-membrane vesicle, the autophagosome, surrounds proteins and organelles to sequester them from the cytosol. A large number of 'Atg' proteins are involved in autophagosome formation, and many have been identified and characterised in *S. cerevisiae*. For example, a Ubl, Atg8, is conjugated to PE lipids of the growing autophagosome. Following completion, the autophagosome fuses with the vacuole/lysosome and releases its inner vesicle into the lumen, where it becomes known as an autophagic body. Enzymes in the lumen of the vacuole/lysosome break down the autophagic body membrane, allowing proteolytic enzymes to break down the proteins and organelles within. Breakdown products can then be 'recycled' to the cytoplasm.

selective autophagy, leading to oxidative stress-induced cell death (Yu et al., 2006). Furthermore, the p62 protein binds polyubiquitinated protein aggregates and directs them to the autophagosome (Pankiv et al., 2007). Organelles can also be targeted for autophagy. For example, mitochondrial autophagy (or ‘mitophagy’) is induced in mammals in response to hypoxia and coenzyme Q deficiency, possibly to prevent excessive ROS generation under these conditions (Rodriguez-Hernandez et al., 2009; Zhang et al., 2008). Mitophagy is also induced in budding yeast when cells are grown in media containing a fermentable carbon source, which can be metabolised by peroxisomes but not by mitochondria (Kanki and Klionsky, 2008). Although the molecular mechanism of mitophagy remains unclear, studies in yeast have shown that mitochondrial proteins such as Uth1 and Aup1 are required for mitophagy (Kissova et al., 2004; Okamoto et al., 2009; Tal et al., 2007). There is also evidence that ubiquitination of dysfunctional mitochondria may trigger mitophagy to protect against oxidative stress in mammalian cells (Geisler et al., 2010; Narendra et al., 2008). Other organelles selectively degraded by autophagy include peroxisomes (‘pexophagy’), which are broken down when *S. cerevisiae* cells are shifted from media containing a fermentable carbon source to media containing a non-fermentable carbon source, making peroxisomes less important for carbon metabolism (Hutchins et al., 1999).

#### 1.1.6.2. *Atg12/Atg8 conjugation*

Studies in *S. cerevisiae* have identified many genes required for autophagy (Tsukada and Ohsumi, 1993). These are now referred to as ‘*atg*’ genes in yeast (Klionsky et al., 2003), and encode proteins involved in autophagosome formation, delivery to the vacuole, and recycling of degradation products. The functions of these proteins have been extensively investigated (Klionsky, 2005; Nakatogawa et al., 2009; Xie and Klionsky, 2007; Yorimitsu and Klionsky, 2005). Significantly, several Atg proteins are involved in a conjugation system involving the UbIs Atg12 and Atg8, which has been well-characterised in *S. cerevisiae* (Geng and Klionsky, 2008) (**Fig. 1.4**). Although Atg12 and Atg8 are very different to other UbIs in terms of amino acid sequence, these autophagic UbIs also have  $\beta$ -grasp folds and are highly-conserved in eukaryotes (Sugawara et al., 2004; Suzuki et al., 2005). Atg12 is synthesised in a mature form, and is conjugated to the Atg5 protein by the E1, Atg7, and the E2,



**Figure 1.4. Autophagic Ubl conjugation pathways.**

The conjugation of Atg12 and Atg8 is key for autophagosome formation in eukaryotes. The E1, Atg7, and the E2, Atg10, facilitate Atg12 conjugation to Atg5 at the pre-autophagosomal structure (PAS). The Atg12-Atg5 complex recruits a third protein, Atg16, and forms multimers. Atg7 can also act as an E1 in the Atg8 conjugation process, transferring Atg8 to another E2, Atg3. Atg8 is finally conjugated to PE lipids during autophagosome formation. Atg8-PE formation requires the Atg12-Atg5-Atg16 complex, which acts as a novel 'E3' by facilitating Atg8 transfer to PE. Levels of Atg8-PE formation have been proposed to control expansion of the autophagosome during its formation. The isopeptidase enzyme Atg4 removes Atg8 from the membrane, and is also required to process precursor Atg8 (prAtg8) into a form that can be activated by Atg7.



Atg10 (Mizushima et al., 1998; Shintani et al., 1999; Tanida et al., 1999). Atg12-Atg5 complexes form multimeric structures which also include Atg16 (Mizushima et al., 1999). Fascinatingly, Atg7 also functions as an E1 in the Atg8 conjugation pathway. Following activation by Atg7, Atg8 is transferred to the E2, Atg3, and finally conjugated to PE lipids on the growing autophagosomal membrane (**Fig. 1.4**) (Ichimura et al., 2000; Kirisako et al., 1999; Kirisako et al., 2000). The ULP, Atg4, is required to process Atg8 to its mature form prior to activation by Atg7, and can also remove Atg8 from PE (Kirisako et al., 2000).

Autophagic Ubl conjugation differs from other ubiquitin/Ubl conjugation pathways in many ways. Firstly, Atg7 functions as an E1 for both Atg12 and Atg8. Secondly, Atg8 conjugation is unique in that a membrane lipid is modified, rather than a protein. Furthermore, Atg12 conjugation is required for Atg8 conjugation, with the Atg12-Atg5-Atg16 complex suggested to function as an 'E3' to promote the formation of Atg8-PE (Hanada et al., 2007; Suzuki et al., 2001). Many structural features of the enzymes in this pathway are also unique (Noda et al., 2009). For example, Atg7 forms a homodimer via a C-terminal domain (Komatsu et al., 2001). In addition, Atg7-Atg3 interactions are mediated by the N-terminal domain of Atg7, rather than a Ubl fold (Taherbhoy et al., 2011), and extensions to the core E2 structure of Atg3 facilitate interactions with Atg7 and Atg8 (Yamada et al., 2007),

Interestingly, although autophagic Ubl conjugation pathways are largely conserved (Geng and Klionsky, 2008), some elements appear to vary between organisms. This is especially true for Atg12 conjugation facilitated by Atg10. While the *Caenorhabditis elegans* homologue of *S. cerevisiae* Atg10 can be identified via amino acid sequence homology (Shintani et al., 1999), this is not the case in *S. pombe* (Mukaiyama et al., 2009) or in mammals (Nemoto et al., 2003; Shintani et al., 1999). Indeed, the mammalian homologue of Atg10 was initially identified using a two-hybrid screen for proteins interacting with Atg5, as this was not possible using a BLAST search alone (Mizushima et al., 2002; Nemoto et al., 2003). Given the substantial variation in amino acid sequence, it is likely that Atg10 homologues have very different tertiary structures. It is also possible that more than one E2 can facilitate Atg12 conjugation in some organisms. Although Atg10 is essential for Atg12 conjugation in *S. cerevisiae*, and Atg10 can facilitate Atg12 conjugation in mammals, small amounts of the Atg12-Atg5 conjugate are found in extracts from mammalian cells lacking Atg10 (Mizushima et al.,



2002; Nemoto et al., 2003). Moreover, the mammalian Atg8 homologue, LC3, is conjugated to PE in the absence of Atg10 (Mizushima et al., 2002). Finally, Atg12 can be conjugated to proteins other than Atg5 in mammalian cells (Radoshevich et al., 2010). It is possible that variations in the structure of Atg10 allow the E2 to facilitate Atg12 conjugation to different proteins in different organisms. It is also possible that mammalian E2s other than Atg10 can facilitate Atg12 conjugation to proteins including Atg5.

The Atg12/Atg8 conjugation pathway is essential for autophagy, and levels of Atg8-PE have been shown to control autophagosome size (Xie et al., 2008). This makes autophagic Ubl conjugation an attractive regulatory target, as any effects on Atg8 conjugation will influence overall autophagic activity. Indeed, the regulation of Atg8 conjugation appears to be an important mechanism in eukaryotes (see Section 1.2.3.1).

#### *1.1.6.3. Autophagy and disease*

The control of autophagy is critical for normal cellular function, and dysfunctional autophagy is often linked to human diseases (Cuervo, 2004; Levine and Kroemer, 2008; Mizushima et al., 2008; Shintani and Klionsky, 2004). In this context, autophagy is considered a ‘double-edged sword’ (Shintani and Klionsky, 2004), as autophagy can prevent disease, but can also contribute to pathogenesis. Autophagy removes damaged proteins and organelles from cells to protect against disease. For example, inhibition of autophagy causes the accumulation of dysfunctional mitochondria in mouse models, resulting in increased ROS generation (Wu et al., 2009). This demonstrates the role of autophagy in protecting cells against excessive ROS generation and oxidative stress, which can result in genetic damage and lead to cancer (see Section 1.2.1.2). Autophagy is also important for the removal of mutant proteins or aggregates to prevent neurodegeneration. For example, mutant Huntingtin protein is removed from cells via autophagy, as p62 binds polyubiquitinated Huntingtin and directs it to the autophagosome (Bjorkoy et al., 2005). Conversely, dysfunctional autophagy can lead to disease, as reviewed elsewhere (Cuervo, 2004; Levine and Kroemer, 2008; Mizushima et al., 2008; Shintani and Klionsky, 2004). For example, autophagy may provide an

alternative energy source for cells growing in low-nutrient environments, which may assist the survival of cancer cells during tumour growth and metastasis. Furthermore, increased autophagic activity may remove chemotherapeutic drugs from tumour cells, again promoting their survival. Excessive autophagic activity may also contribute to the death of neuronal cells during neurodegeneration. Thus, it is vital that we understand the molecular mechanisms of autophagy regulation in health and disease, which may be exploited to increase or decrease autophagic activity during therapy.

#### *1.1.6.4. Regulation of autophagy*

Although autophagy occurs at a basal level in eukaryotic cells, the process is upregulated during nitrogen starvation. This is achieved via multiple mechanisms. For example, studies in *S. cerevisiae* have shown that TOR inactivation during nitrogen starvation is important for the induction of autophagy. When TOR activity is decreased, the Atg13 protein becomes hypophosphorylated, thus increasing its affinity for the key kinase, Atg1. A strong Atg1-Atg13 interaction activates Atg1, leading to increased autophagic activity, although the substrates of Atg1 are unknown (Kamada et al., 2000; Noda and Ohsumi, 1998). In a second mechanism, the mammalian SAPK, JNK, becomes activated during nitrogen starvation and phosphorylates the Bcl-2 protein, causing Bcl-2 to dissociate from the Atg6 homologue Beclin-1. Dissociation of Bcl-2 allows Beclin-1 to facilitate the recruitment of Atg proteins to the site of autophagosome formation, thus increasing autophagic activity (Geeraert et al., 2010; Pattingre et al., 2005; Wei et al., 2008). Indeed, the best-characterised mechanisms of autophagy induction involve Atg1 and Atg6/Beclin-1 complexes (Pattingre et al., 2008).

Additionally, many well-known signalling pathways and molecules have been shown to inhibit autophagy. Similar to TOR, PKA inhibits autophagy in *S. cerevisiae* through phosphorylation of Atg13, helping to keep autophagic activity low (Budovskaya et al., 2004; Stephan et al., 2009). However, PKA activity is decreased during carbon starvation due to low intracellular cAMP production, allowing autophagy to break down cellular components and thus provide an alternative source of carbon (Budovskaya et al., 2004; Stephan et al., 2009). In mammalian cells, pro-growth proteins such as

Akt/PKB and the transcription factor NF $\kappa$ B are required for optimal TOR activity under homeostatic conditions, again maintaining a low basal level of autophagic activity (Arico et al., 2001; Djavaheri-Mergny et al., 2006). Determining the relative contributions of these pathways to the control of autophagy under different circumstances remains a priority of research. Moreover, there are likely to be other molecular mechanisms of autophagy regulation that remain unknown.

Significantly, there is accumulating evidence that ROS generation induces autophagy in eukaryotes, possibly to remove oxidatively-damaged molecules from cells (Azad et al., 2009; Bensaad et al., 2009; Chen et al., 2007, 2008b; Huang et al., 2009). How autophagy is induced during oxidative stress remain largely unclear. However, deacetylation of proteins involved in autophagic Ubl conjugation, such as Atg5, Atg7 and Atg8, correlates with increased autophagic activity in mammalian cells, and can be mediated by Sirt1, which is activated in response to accumulation of NAD<sup>+</sup> during oxidative stress (Lee et al., 2008; Lee and Finkel, 2009; Salminen and Kaarniranta, 2009).

#### **1.1.7. Introduction to redox-regulation of ubiquitin/Ubl conjugation**

During its life cycle, a cell may experience changes to its external environment. For example, changes in the environment of a cell may result in increased ROS exposure. Cellular antioxidant defences may become insufficient if ROS exposure increases; a condition known as oxidative stress, which is frequently associated with disease (see Section 1.2.1.2). Thus, it is highly important for the cell to be able to adapt and respond to these changes in order to remain viable. This is particularly true for unicellular organisms, such as yeast. Adaptation to different conditions will often involve modulation of key cellular processes. As described above, ubiquitin/Ubl conjugation pathways are established as key regulators of a range of cellular proteins and pathways. Thus, regulation of ubiquitin/Ubl conjugation represents an attractive mechanism allowing cells to regulate fundamental cellular processes in response to stress.

It has been reported that ubiquitin/Ubl conjugation is induced in some cases during oxidative stress. Indeed, exposure to  $\text{H}_2\text{O}_2$  increases the transcription of many genes encoding E2s and E3s in both lower and higher eukaryotes (Chen et al., 2008a; Vandenbroucke et al., 2008). Although the proteins targeted for ubiquitination by these E2s and E3s are largely unknown, increasing their levels or activity could be important for resistance to oxidative stress. Conversely, the increased ubiquitination of specific proteins may result in disease. For example, prolonged exposure of skeletal muscle cells to  $\text{H}_2\text{O}_2$  induces the expression of a limited number of E2s, such as E2<sub>14K</sub>, and E3s, such as MuRF1, which have been implicated in muscle protein catabolism (Li et al., 2003b). Thus, while oxidative stress-induced ubiquitination may protect cells against oxidative damage, or contribute to pathologies such as muscle wasting diseases. Furthermore, oxidative stress has been reported to increase the conjugation of UbIs such as Urm1 (Goehring et al., 2003a; Van der Veen et al., 2011; Zhou et al., 2004).

Conversely, ubiquitin/Ubl conjugation can also be inhibited during oxidative stress. ‘Redox regulation’ is an important sensing mechanism involving oxidation of specific proteins by ROS such as  $\text{H}_2\text{O}_2$ . The resulting changes in protein activity can allow cells to sense and respond to oxidative stress (although redox regulation is also important under homeostatic conditions, as described in Section 1.2). Redox regulation often involves the oxidation of cysteine residues, leading to changes in protein function. It is now established that  $\text{H}_2\text{O}_2$  can inhibit some ubiquitin/Ubl conjugation pathways by oxidising the catalytic cysteine residues of specific E1s and E2s. However, it is largely unclear how redox regulation of ubiquitin/Ubl conjugation impacts upon oxidative stress responses in eukaryotes. It is also unclear whether such regulatory mechanisms are conserved or species-specific. In the next section, the nature, basis and importance of redox regulation will be outlined, and well-characterised examples of redox regulation in prokaryotes and eukaryotes will be described. Redox regulation of ubiquitin/Ubl conjugation pathways will then be discussed.

## 1.2. Oxidative Stress and Signalling by Reactive Oxygen Species

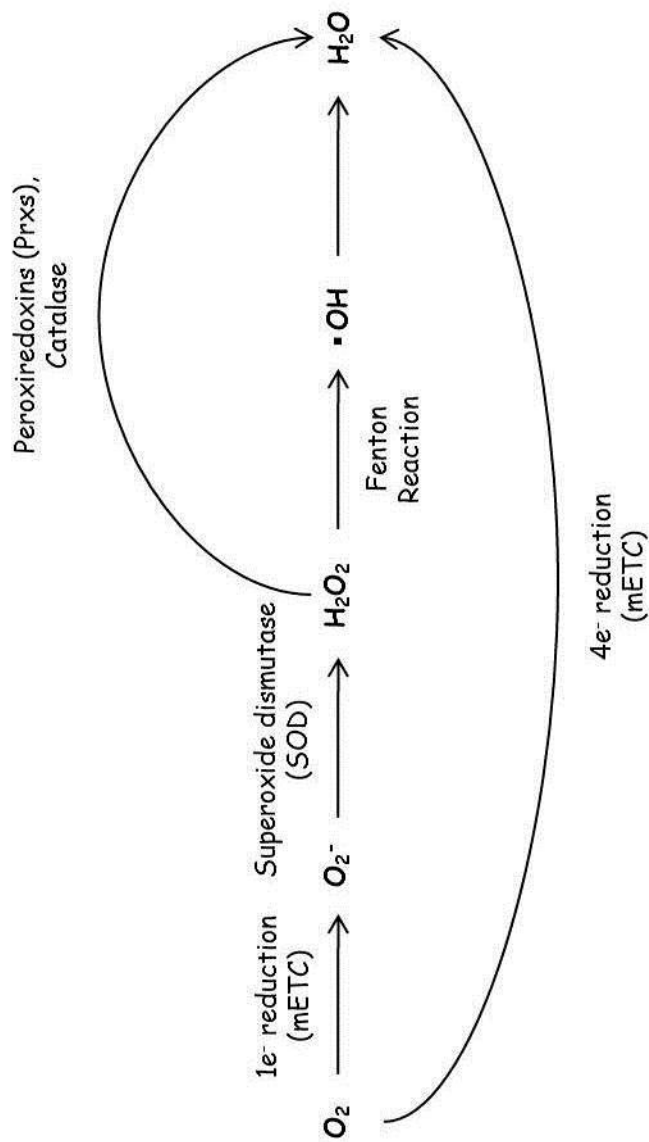
### 1.2.1. ROS and oxidative stress

#### *1.2.1.1. ROS and their generation*

ROS are produced by the incomplete reduction of oxygen to water (**Fig. 1.5**). A single-electron reduction of oxygen yields  $O_2^-$ , which is further reduced to  $H_2O_2$ . Further reduction can be catalysed by metal ions (known as the Fenton reaction), producing the highly reactive  $OH^\cdot$ . Many more oxygen-containing radicals can be produced, particularly following reaction of ROS with the signalling molecule NO, causing generation of RNS. However, it is  $O_2^-$ ,  $H_2O_2$  and  $OH^\cdot$  that will be considered here.

ROS can be produced by both endogenous and exogenous sources. Mitochondria represent a large source of cellular ROS, as aerobic metabolism involving the mETC makes ROS generation inevitable. Indeed, it has been estimated that 1-2% of mETC electrons can generate  $O_2^-$  (Boveris and Chance, 1973; Halliwell and Gutteridge, 1990), although recent studies suggest the figure may be lower (Staniek and Nohl, 2000). It has been proposed that mETC complex III and an intermediate in coenzyme Q regeneration are the primary sources of electron leakage (Finkel and Holbrook, 2000; Turrens, 1997). Peroxisomes and the endoplasmic reticulum have also been identified as endogenous sources of ROS, and phagocytes use NADPH oxidase enzymes to generate ROS as part of host defence mechanisms (Adams et al., 1982). Additional, exogenous sources of ROS include UV light (Cerutti, 1985), ionising radiation (Pollycove and Feinendegen, 2003) and cigarette smoke (Cosgrove et al., 1985).

Additionally, ROS are actively generated as part of signal transduction pathways. For example, in higher eukaryotes,  $H_2O_2$  is produced downstream of RTKs, including the PDGF receptor (Sundaresan et al., 1995) and the EGF receptor (Bae et al., 1997). ROS generation downstream of RTKs is dependent on PI3K (Bae et al., 2000), and evidence



**Figure 1.5. ROS are generated intracellularly, and can be removed from the cell by antioxidant enzymes.**

The mitochondrial electron transport chain (mETC) generates ATP during oxidative phosphorylation, a biochemical process involving the reduction of oxygen to water. The superoxide anion ( $\text{O}_2^-$ ) is often generated at the mETC due to single-electron reduction of oxygen. Superoxide dismutase (SOD) enzymes can reduce  $\text{O}_2^-$  to hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), a less reactive molecule which also functions in cell signalling. It is important that  $\text{H}_2\text{O}_2$  is rapidly removed from the cell, as Fenton chemistry allows further reduction by metal ions, yielding the hydroxyl radical ( $\bullet\text{OH}$ ), which is highly reactive and able to cause oxidative damage to many cellular components. Many highly abundant antioxidant enzymes, including peroxiredoxins (Prxs) and catalase, can remove  $\text{H}_2\text{O}_2$  from the cell using redox mechanisms.

suggests that ROS are produced by Rac1-mediated activation of Nox enzymes downstream of PI3K (Park et al., 2004; Rhee et al., 2000; Rhee et al., 2003). Generation of ROS by Nox enzymes is implicated in a range of processes, and dysfunction can lead to disease (Lambeth, 2004). However, there is also evidence that receptor-ligand interactions somehow trigger extracellular generation of H<sub>2</sub>O<sub>2</sub>, which then diffuses into the cell (DeYulia et al., 2005).

#### *1.2.1.2. ROS and disease*

ROS can cause oxidative damage to many cellular components, including DNA, proteins and lipids, and this has long been associated with many human diseases. For example, direct oxidation of DNA can result in the formation of oxo<sup>8</sup>dG adducts, which can lead to transversion mutations. Oxidative damage to nuclear DNA has been implicated in cancer and ageing (Finkel and Holbrook, 2000). As mitochondria produce significant quantities of ROS, mtDNA is also susceptible to oxidative damage, which can cause mitochondrial dysfunction, and thus increase ROS generation yet further (Finkel and Holbrook, 2000). Indeed, accumulation of damaged mitochondria has been linked to increased ROS production and shortened lifespan in fission yeast (Zuin et al., 2008), and recent evidence suggests that increased mitochondrial ROS generation causes budding yeast to decrease mitochondrial biogenesis, possibly to protect against oxidative stress (Chevtzoff et al., 2010). Proteins are also potential targets of ROS, with oxidation of the peptide backbone leading to peptide bond cleavage and protein-protein cross-links (Berlett and Stadtman, 1997). Amino acid side chains can also be irreversibly oxidised, and carbonyl derivatives can be formed following protein oxidation (Berlett and Stadtman, 1997). The resulting dysfunction in cellular pathways can lead to diseases such as Alzheimer's disease (Butterfield and Boyd-Kimball, 2004; Kadowaki et al., 2005), diabetes (Imoto et al., 2006) and cancer (Oberley, 2002). Moreover, lipid oxidation produces aldehyde breakdown products which can react with DNA, leading to genetic damage (Beckman and Ames, 1997).

ROS have also been linked to the ageing process. Indeed, the classic 'free radical theory' of ageing states that the accumulation of oxidative damage to cellular

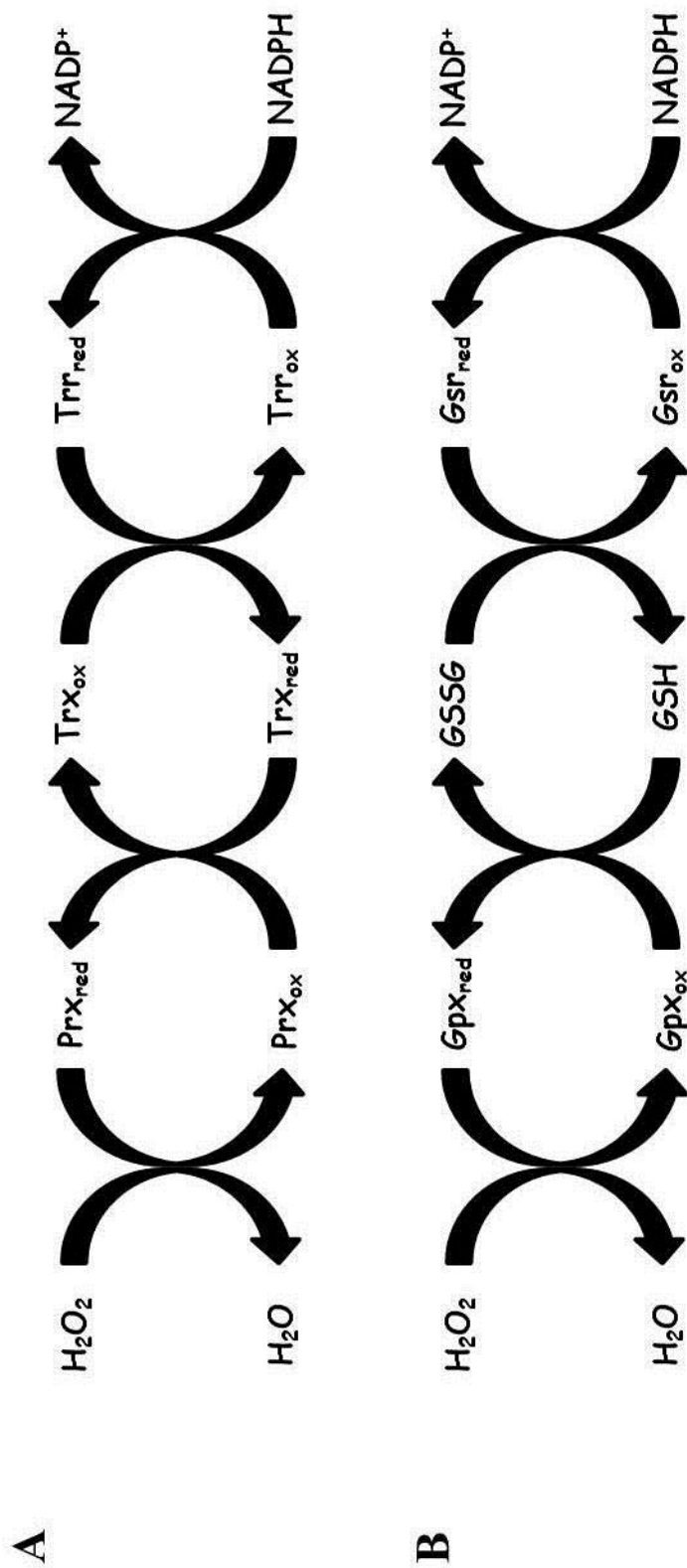
components is central to the ageing process (Harman, 1956). Consistent with this theory, studies in a range of organisms have demonstrated that levels of oxidised macromolecules increase with age (Balaban et al., 2005). Moreover, mutations in highly-conserved pathways can decrease the cellular capacity to remove ROS, and have been linked to a reduction in life span in numerous vertebrate and invertebrate model organisms (Balaban et al., 2005). Given that oxidative damage is involved in ageing and the pathogenesis of disease, it is critical that cells have antioxidant mechanisms that remove ROS.

#### *1.2.1.3. ROS removal by antioxidants*

Cells employ a range of mechanisms to remove ROS and protect against oxidative damage. There are many non-enzymatic ROS scavengers present in cells, including vitamins A and E. However, there are also many enzymatic systems that can remove ROS from cells. As shown in **Fig. 1.5**, SOD enzymes catalyse the reduction of  $O_2^-$  to the comparatively stable molecule  $H_2O_2$ . The cellular localisation of SOD enzymes is frequently regulated to increase their efficiency. For example, mammalian SOD2 localises to the mitochondria to remove  $O_2^-$  generated by the mETC (Balaban et al., 2005).

Following the generation of  $H_2O_2$ , the metal ion-catalysed Fenton reaction can produce dangerous  $OH\cdot$  radicals (**Fig. 1.5**). Consequently, it is crucial that cells have a range of enzymes that catalyse the two-electron reduction of  $H_2O_2$  to water. One such enzyme is catalase, which utilises a heme group to reduce  $H_2O_2$ . Catalase tends to localise to the peroxisome, suggesting that it has a major role in removal of ROS generated by peroxisomes. Other enzymes include glutathione peroxidases and thioredoxin peroxidases (Prxs), which reduce  $H_2O_2$  using catalytic selenocysteine and cysteine residues, respectively. These residues are maintained in the reduced form by either GSH or Trx, which are themselves reduced by enzymes dependent on the electron donor NADPH for their activity (**Fig. 1.6**). Prxs are also able to remove alkyl hydroperoxides such as *t*-BOOH from the cell (Rhee et al., 2003). Like SODs, Prxs may be targeted to specific sites within the cell to increase the efficiency of ROS removal. For example,





**Figure 1.6. Peroxidase enzymes remove  $H_2O_2$  from the cell, and are reactivated as part of key redox cascades.**

**A.** 2-Cys Prxs use a peroxidatic cysteine residue to remove  $H_2O_2$  from the cell. The reduction of  $H_2O_2$  to water causes the formation of a disulfide bond between the oxidized peroxidatic cysteine and the resolving cysteine of another Prx monomer. Disulfide bonds can be reduced by Trx, which becomes oxidized in the process. Oxidized Trx is reduced back to the original form by thioredoxin reductase (Trx) enzymes. The electron donor NADPH is generated by the pentose phosphate pathway, and is required to reactivate Trx. **B.** Gpxs can remove  $H_2O_2$  using a mechanism similar to that of 2-Cys Prx. Oxidized Gpx is reactivated by GSH, which is maintained in the reduced state by GSH reductase (Gsr) and NADPH.

mammalian PrxII can be targeted to the cell membrane, PrxIII localises to the mitochondria, and PrxIV is located in the ER (Choi et al., 2005; Rhee et al., 2012; Veal et al., 2007).

Due to constant ROS generation within cells, antioxidant enzymes are highly expressed under homeostatic conditions. Indeed, it has been estimated that PrxI and PrxII may account for as much as 1% of cellular protein in mammalian cells (Chae et al., 1999). However, cellular antioxidant defences may become overwhelmed if ROS generation increases; a condition known as oxidative stress. It is therefore essential that cells are able to increase their antioxidant capacity in response to stress. MAPK pathways are highly conserved phosphorylation cascades that regulate the cellular response to various stimuli, including oxidative stress (Marshall, 1994; Waskiewicz and Cooper, 1995). Specific stimuli trigger the activation of a MAPKKK, leading to the phosphorylation of a second enzyme, the MAPKK, which finally activates the MAPK. SAPKs, such as mammalian p38 and JNK, are activated in response to oxidative stress, and facilitate increases in the antioxidant capacity of cells (Waskiewicz and Cooper, 1995). It is essential that SAPK activity is regulated appropriately, as dysfunction of these pathways can lead to disease (Kennedy and Davis, 2003; Takeda and Ichijo, 2002).

As outlined above, it is critical that low ROS levels are maintained in order to limit cellular damage and prevent disease. To this end, cells employ a range of antioxidant molecules and mechanisms, and are able to respond to changes in redox status. However, it has become clear in recent years that ROS – such as  $\text{H}_2\text{O}_2$  – have roles as signalling molecules within the cell. Indeed, it is often through the oxidation of specific proteins that cells sense increases in ROS levels and trigger oxidative stress responses. The basis for the specificity of protein oxidation, and how oxidation regulates protein function, will now be discussed.

### **1.2.2. Signalling by ROS**

$\text{H}_2\text{O}_2$ ,  $\text{O}_2^-$  and  $\text{OH}^\cdot$  are the major ROS discussed in this review. Of these,  $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$  are established as signalling molecules. Although other ROS and RNS, such as NO (Levonen et al., 2001) can function in signalling, they are outside the scope of this literature review.

Although  $\text{OH}^\cdot$  reacts with cellular components indiscriminately,  $\text{O}_2^-$  has a degree of selectivity towards metal ion-binding sites, particularly iron-sulphur clusters (D'Autreaux and Toledano, 2007). Oxidation of an iron-sulphur cluster releases iron, causing large conformational changes within the structure of the target protein, thus regulating its activity (D'Autreaux and Toledano, 2007). For example, the prokaryotic transcription factor SoxR contains an iron-sulphur cluster that is sensitive to oxidation by  $\text{O}_2^-$  (Hidalgo and Demple, 1994). Oxidation of the iron-sulphur cluster activates SoxR, allowing the transcription factor to induce the expression of oxidative stress response genes, including SOD enzymes required for the removal of  $\text{O}_2^-$  (Liochev and Fridovich, 1992).

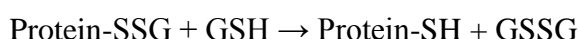
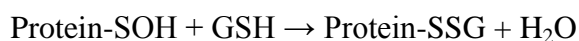
While  $\text{O}_2^-$  has important signalling properties, the use of  $\text{H}_2\text{O}_2$  as a signalling molecule is far more widespread. In contrast to the unstable, highly reactive  $\text{O}_2^-$  and  $\text{OH}^\cdot$ ,  $\text{H}_2\text{O}_2$  is uncharged, long-lived and can diffuse across membranes, making it ideal as a signalling molecule. Indeed, redox regulation by  $\text{H}_2\text{O}_2$  is an important regulatory mechanism in both higher and lower organisms. In this section, the nature of  $\text{H}_2\text{O}_2$  signalling will be discussed, and the importance of  $\text{H}_2\text{O}_2$  signalling in oxidative stress responses will be outlined. Redox regulation of ubiquitin/Ubl conjugation will also be described.

#### *1.2.2.1. $\text{H}_2\text{O}_2$ as a signalling molecule*

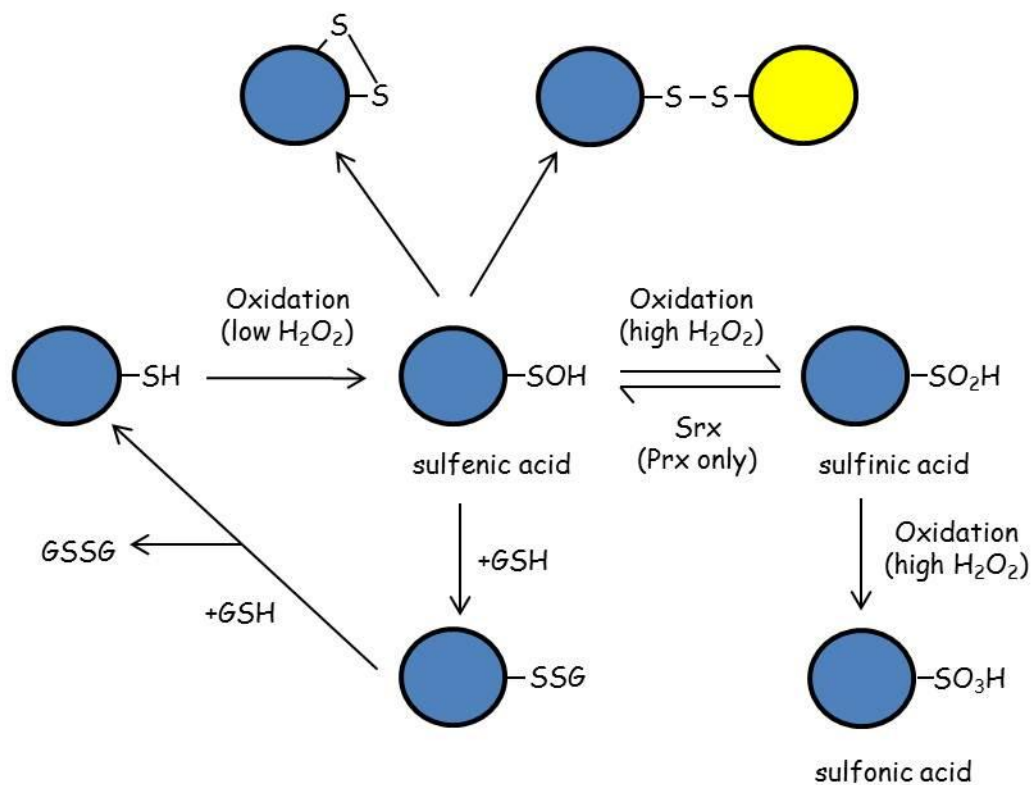
It has been shown that  $\text{H}_2\text{O}_2$  can selectively oxidise cysteine residues, leading to redox regulation of protein activity, stability, interactions or function (D'Autreaux and Toledano, 2007; Rhee, 2006; Veal et al., 2007). There are many similarities between phosphorylation and cysteine oxidation as post-translational modifications regulating protein activity. They are both highly specific and reversible (Filomeni et al., 2005), and are targeted to certain sites within proteins. In the case of cysteine oxidation, specificity

is conferred by the microenvironments of specific cysteine residues. While typical cysteine residues have a pKa of ~8.5, permanent or transient microenvironments can lower the relative pKa of a cysteine thiol, leading to deprotonation at neutral pH, and the formation of a negatively-charged thiolate anion (**Fig. 1.7**) (Rhee et al., 2000). Backbone amide nitrogens and positively-charged, basic amino acid residues, such as lysine and arginine, are often involved in stabilising thiolate anions (Barford, 2004). It is these deprotonated cysteines that are preferentially oxidised by H<sub>2</sub>O<sub>2</sub>; a process initiated by nucleophilic attack on the peroxide bond by the thiolate anion (Barford, 2004; D'Autreaux and Toledano, 2007). Cysteine oxidation causes the formation of sulfenic acid and, consequently, often leads to the formation of intramolecular or intermolecular disulfide bonds (**Fig. 1.7**). Disulfide bond formation is central to redox regulation of proteins, as will be described below. It is also important to note that hyperoxidation of cysteine residues is possible, and can generate sulfinic or sulfonic acid forms (**Fig. 1.7**).

Importantly, GSH can form transient disulfide bonds with oxidised cysteine residues, preventing both promiscuous disulfide bond formation and hyperoxidation of cysteine residues (Ghezzi, 2005). Glutathionylation can be resolved to yield a reduced cysteine and GSSG, as shown in **Fig. 1.7** and below:

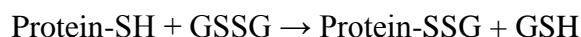


GSSG can then be recycled to GSH by glutathione reductases in an NADPH-dependent manner (**Fig. 1.6**). High concentrations of GSH are important in maintaining a reducing state within the cytosol. However, the compartmentalisation of glutathione into 'pools' causes some cellular compartments to be slightly more oxidising. Such compartments include the ER, which is important for the formation of native disulfides during protein folding (Chakravarthi et al., 2006). It has been proposed that the susceptibility of cysteines to glutathionylation is largely dependent on three factors: (i) the redox-sensitivity of the cysteine, (ii) the accessibility of the cysteine, and (iii) the protein environment, as a neighbouring cysteine residue may make intramolecular disulfide formation more likely than an intermolecular bond with GSH (Ghezzi, 2005). It is also



**Figure 1.7. Cysteine thiols can exist in the reduced form, or can be oxidised to varying degrees.** In the majority of cases, cysteine thiols are maintained in the reduced form ( $\text{-SH}$ ) at cytosolic pH, and have fairly high pKa values, typically  $\text{pH} \sim 8.5$ . Oxidation of cysteine thiols by  $\text{H}_2\text{O}_2$  generates sulfenic acid groups ( $\text{-SOH}$ ), which are able to form disulfide bonds with neighbouring cysteines within the protein and/or associated proteins. Disulfide bonds are also formed with the cellular reductant GSH. Glutathionylation of proteins can be reversed by disulfide exchange, returning the cysteine to its reduced form and liberating GSSG. If cells are exposed to high concentrations of  $\text{H}_2\text{O}_2$ , cysteine thiols can be ‘hyperoxidised’ to sulfinic acid ( $\text{-SO}_2\text{H}$ ) or sulfonic acid ( $\text{-SO}_3\text{H}$ ). In the majority of cases, hyperoxidation of cysteines is irreversible. Thus, disulfide bond formation can function as a protective mechanism, preventing irreversible oxidation of cysteine residues. However, sulfinic acid formation can be reversed by Srx enzymes, allowing ‘reactivation’ of these antioxidant enzymes following exposure to high levels of  $\text{H}_2\text{O}_2$ .

possible that a decrease in the GSH:GSSG ratio can result in glutathionylation (Ghezzi, 2005). However, it has also been proposed that physiological changes to cellular GSH:GSSG would be insufficient to promote modification of redox-insensitive thiols (Gallogly and Mieyal, 2007):



Significantly, cysteine oxidation by H<sub>2</sub>O<sub>2</sub> may regulate protein function not only by triggering intramolecular disulfide formation, but also via glutathionylation of cysteines. For example, glutathionylation of the catalytic cysteine of PTP1B inhibits the phosphatase following cysteine oxidation (Barrett et al., 1999; Lee et al., 1998b). In addition, glutathionylation of DNA-binding domains inhibits the activity of transcription factors such as NFκB and AP-1 (Filomeni et al., 2005). Furthermore, glutathionylation and deglutathionylation of specific proteins can be catalysed by glutathione-S-transferases and Grxs, respectively (Gallogly and Mieyal, 2007), and glutathione-S-transferases function in cellular detoxification by catalysing the glutathionylation of toxic compounds (Salinas and Wong, 1999).

#### *1.2.2.2. Redox regulation of prokaryotic transcription factors*

Unicellular organisms such as bacteria are exposed to a wide range of different conditions and stresses, in contrast to the majority of cells in multicellular organisms which are maintained in relatively homeostatic conditions. The ability of lower organisms to sense and respond to fluctuations in ROS levels is therefore highly important for their survival. In bacterial cells, it has been shown that the transcription of particular genes is induced during oxidative stress via redox regulation of specific transcription factors. In addition to SoxR regulation by O<sub>2</sub><sup>-</sup>, Storz *et al.* demonstrated that the *Escherichia coli* transcription factor OxyR is oxidised at specific cysteine residues by H<sub>2</sub>O<sub>2</sub>, resulting in a conformational change that causes OxyR to switch from its function as a repressor to an inducer of antioxidant genes (Storz et al., 1990). It has since been shown that an intramolecular disulfide bond is formed following cysteine oxidation, leading to this switch in OxyR function (Choi et al., 2001; Zheng et al.,

1998). Interestingly, cysteine oxidation can also result in glutathionylation of OxyR (Kim et al., 2002). Glutathionylation and disulfide formation appear to trigger different conformational changes within OxyR, which may allow the transcription factor to regulate the expression of different genes in response to different oxidative modifications (Kim et al., 2002). This may allow OxyR to mediate distinct transcriptional responses to different levels of oxidative stress, and in response to different stress agents. The modulation of transcriptional responses in response to different levels of stress is also observed in eukaryotes such as *S. pombe* (see Section 1.3).

OxyR is an example of how redox regulation of protein activity can influence oxidative stress responses. Many eukaryotic proteins are also known to be redox-sensitive. Redox regulation of enzymes such as PTPs and Prxs is particularly well-characterised in eukaryotes and, like redox regulation of OxyR, this can be important for resistance to oxidative stress. Redox regulation of specific eukaryotic proteins, and how redox regulation can impact on oxidative stress responses in eukaryotes, is discussed below.

#### *1.2.2.3. Redox regulation of PTPs*

A classic example of redox regulation by  $\text{H}_2\text{O}_2$  is the reversible inactivation of PTPs (Denu and Dixon, 1998). The active site of PTPs includes a cysteine thiol with a low pKa, allowing stable deprotonation of the cysteine at neutral pH (Denu and Dixon, 1998). Cysteine deprotonation is required for the formation of a thiol-phosphate intermediate as part of the dephosphorylation mechanism catalysed by PTPs (Rhee et al., 2000; Rhee et al., 2003). However, deprotonation also renders the cysteine residue highly sensitive to oxidation by  $\text{H}_2\text{O}_2$ . Indeed, it has been shown that phosphatases such as PTP1B (Lee et al., 1998b) and PTEN (Lee et al., 2002) are inactivated by  $\text{H}_2\text{O}_2$  in mammalian cells. Oxidative inactivation of phosphatases promotes signalling downstream of RTKs by preventing dephosphorylation of these receptors and by decreasing hydrolysis of the membrane phospholipid  $\text{PIP}_3$ , which is important for signalling downstream of RTKs (Rhee et al., 2005).

Cdc25C is another PTP that can be inactivated by  $H_2O_2$ . Cdc25 phosphatases promote entry into mitosis by removing inhibitory phosphates from Cdks. Oxidation of the catalytic cysteine of Cdc25C causes a change in protein conformation, resulting in nuclear export and degradation of the phosphatase (Savitsky and Finkel, 2002). Thus, redox regulation of protein activity seems to be an important mechanism controlling cell cycle progression. Indeed, oscillations of ROS levels have been observed throughout the cell cycle, and have resulted in the cell cycle being referred to as a ‘redox cycle’ (Burhans and Heintz, 2009). As there are many PTPs acting on many different substrates in eukaryotes, redox regulation of PTP activity may have a multitude of effects on cellular pathways and processes.

#### *1.2.2.4. Redox regulation of eukaryotic Prx*

As outlined above, Prxs are able to reduce  $H_2O_2$  to water. Prxs have been divided into three subgroups: typical 2-Cys Prx, atypical 2-Cys Prx and 1-Cys Prx (Cash et al., 2007). In the case of typical 2-Cys Prx, the ‘peroxidatic’ cysteine residue of the enzyme reduces  $H_2O_2$  and becomes oxidised to a sulfenic acid form (**Fig. 1.7**). Structural studies indicate that the redox-sensitivity of the peroxidatic cysteine is conferred by its proximity to backbone amide groups and arginine and threonine residues (Barford, 2004). Oxidation leads to the formation of a disulfide bond between the peroxidatic cysteine and the ‘resolving’ cysteine of another Prx monomer, leading to homodimerisation (Rhee et al., 2003; Rhee et al., 2005). These dimers are then resolved by Trx (**Fig. 1.7**).

2-Cys Prx are highly conserved antioxidant enzymes found in both prokaryotes and eukaryotes. However, interestingly, it has become clear that eukaryotic 2-Cys Prx are more sensitive to hyperoxidation of their peroxidatic cysteine residues than their prokaryotic counterparts (Wood et al., 2003a; Wood et al., 2003b), although recent evidence suggests that some bacterial Prx are also sensitive to hyperoxidation (Pascual et al., 2010). It has been proposed that structural properties of eukaryotic 2-Cys Prx, including a C-terminal extension helix, increase the stability of the oxidised form of the enzyme, thus delaying disulfide bond formation and allowing further oxidation of the



peroxidatic cysteine (Wood et al., 2003a). Formation of a sulfinic acid group effectively inactivates the enzyme, as disulfide formation is prevented. It was initially thought that hyperoxidation of eukaryotic 2-Cys Prx caused irreversible inactivation of the enzyme. However, it has since been shown that Srx enzymes are able to reverse this process (**Fig. 1.7**) (Biteau et al., 2003; Rhee et al., 2007; Veal et al., 2007).

The sensitivity of eukaryotic 2-Cys Prx to hyperoxidation led to the proposal that this is important for H<sub>2</sub>O<sub>2</sub> signalling in eukaryotes. Low concentrations of H<sub>2</sub>O<sub>2</sub> will be removed by Prx and other antioxidant enzymes. However, higher concentrations can lead to inactivation of 2-Cys Prx, thus allowing a build-up of H<sub>2</sub>O<sub>2</sub> which acts in signal transduction. This reasoning formed the basis of the ‘floodgate model’, whereby inactivation of 2-Cys Prx allows H<sub>2</sub>O<sub>2</sub> to act as a signalling molecule at relatively low concentrations (Wood et al., 2003a). Importantly, inactivation of 2-Cys Prx need not affect the whole cell. For example, H<sub>2</sub>O<sub>2</sub> produced by Nox enzymes following RTK activation inactivates 2-Cys Prx in the vicinity of the receptor, which allows the accumulation of H<sub>2</sub>O<sub>2</sub> necessary to inactivate PTPs near the cell membrane. This prevents the immediate dephosphorylation of RTKs by PTPs, allowing propagation of the signal (Rhee et al., 2005). However, as H<sub>2</sub>O<sub>2</sub> diffuses into the cell, the relative concentrations of the oxidant will become lower, leading to removal by Prx and other antioxidant enzymes. This would be expected to limit erroneous signalling by H<sub>2</sub>O<sub>2</sub>, and prevent decomposition to the highly-reactive OH $\cdot$ . Moreover, relocalisation of 2-Cys Prx to RTK sites after receptor activation may be important for signal inactivation through removal of H<sub>2</sub>O<sub>2</sub> (Choi et al., 2005). Prx activity can also be regulated by phosphorylation during different phases of the cell cycle (Chang et al., 2002) and in different locations within the cell (Woo et al., 2010), adding extra complexity to the control of redox signalling by 2-Cys Prx. Fascinatingly, these enzymes are also able to function as molecular chaperones following oxidation (Jang et al., 2004), allowing 2-Cys Prx to reverse protein aggregation and misfolding during oxidative stress. In addition, it is now established that eukaryotic cells can tailor their responses to different levels of oxidative stress by utilising the oxidation state of 2-Cys Prx as a sensor of intracellular H<sub>2</sub>O<sub>2</sub> concentrations. This has been described as part of oxidative stress response mechanisms in organisms such as the fission yeast, *S. pombe* (see Section 1.3).

#### 1.2.2.5. Further examples of redox regulation in higher eukaryotes

As discussed above, ROS signalling is important for oxidative stress responses in lower organisms. An important component of this response is the oxidation of regulatory proteins which influence transcriptional responses and promote survival or adaptation to oxidative stress (further examples in *S. pombe* are discussed in Section 1.3). This is also the case in multicellular organisms. However, in multicellular organisms, ROS can function as signalling molecules to regulate a much wider range of processes than just oxidative stress responses, as appears to be the case in unicellular organisms such as bacteria and yeast. For example, NADPH oxidase-dependent generation of ROS causes increases in intracellular calcium in plant root cells, leading to cell growth (Foreman et al., 2003). Evidence suggests that this calcium influx may result from activation of divalent cation channels by  $\text{OH}^\cdot$  (Foreman et al., 2003), although how this is achieved remains unclear. Furthermore, differentiation of cardiomyocytes from embryonic stem cells is driven by ROS signalling (Li et al., 2006). In this case, Nox enzymes generate ROS that trigger a transcriptional programme promoting cardiomyocyte development, possibly through p38 MAPK and the MEF2C transcription factor (Li et al., 2006). Redox signalling is also crucial for angiogenesis, with evidence suggesting that ROS promote tube formation, growth and migration of vascular endothelial cells (Ushio-Fukai, 2006). Although redox-regulated proteins remain unknown in this instance, it has been proposed that redox regulation of angiogenesis may involve (i) inactivation of phosphatases to promote VEGF signalling, (ii) activation of Akt and p38 pathways, and (iii) oxidation and activation of transcription factors influencing cell growth (Ushio-Fukai, 2006). The regulation of so many different processes by ROS has led to the identification of many more redox-regulated proteins in higher eukaryotes. A comprehensive list of redox-regulated proteins, and the effects of their oxidation, can be found elsewhere (Veal et al., 2007). However, a limited number of well-characterised redox targets will now be described.

ASK1 activation during oxidative stress is a classic example of redox regulation of protein activity in higher eukaryotes. ASK1 is a MAPKKK functioning upstream of the mammalian SAPKs p38 and JNK (Ichijo et al., 1997), with roles in many processes including  $\text{TNF}\alpha$ - and ROS-induced apoptosis (Ichijo et al., 1997; Tobiume et al., 2001). Under homeostatic conditions, Trx is bound to the N-terminal region of ASK1, preventing activation of ASK1 and leading to proteasomal degradation of the

MAPKKK (Liu and Min, 2002; Saitoh et al., 1998). However, under oxidative stress conditions, Trx becomes oxidised and dissociates from ASK1, allowing multimerisation and activation of the MAPKKK and leading to activation of the SAPK pathway (Nadeau et al., 2007; Saitoh et al., 1998). Dissociation of Trx also allows the recruitment of key factors required for ASK1 activation, including TRAF2 and TRAF6 (Noguchi et al., 2005). This may not be the only instance in which Trx functions as a 'redox sensor' to regulate kinase activity; indeed, Trx has been linked to the regulation of many other protein kinases (Fujino et al., 2006). Furthermore, there is evidence that JNK phosphatases are inactivated by ROS, and that this may contribute to activation of the SAPK (Kamata et al., 2005). Thus, ROS can activate SAPK pathways in higher eukaryotes via Trx-dependent and Trx-independent mechanisms, thus influencing a range of cellular processes, including apoptosis (Filomeni et al., 2005; Matsuzawa and Ichijo, 2008).

Additionally, transcription factors such as the tumour suppressor p53 are regulated by oxidation and glutathionylation (Filomeni et al., 2005; Veal et al., 2007). Interestingly, p53 target genes encode both antioxidants and pro-oxidants, and p53 has been shown to have antioxidant functions during mild oxidative stress and pro-oxidant functions as stress becomes more severe (Sablina et al., 2005). It was proposed that p53 activates the expression of highly-responsive antioxidant genes during mild stress, while less-responsive pro-oxidant genes are induced during severe stress due to increased levels of p53 (Liu et al., 2008; Sablina et al., 2005). However, ROS may also regulate p53 activity more directly. The p53 protein has redox-sensitive cysteine residues in the DNA-binding domain (Rainwater et al., 1995), and intramolecular disulfide formation has been reported to prevent binding of p53 to recognition sequences (Hainaut and Milner, 1993; Sun et al., 2003). Furthermore, it has been reported that cysteine oxidation alters the affinity of p53 for some recognition sequences, but not others (Buzek et al., 2002). Therefore, it is possible that oxidation of p53 regulates the genes that can be regulated by the transcription factor, favouring the expression of pro-oxidant genes and possibly preventing the expression of anti-oxidant genes. Additionally, p53 is glutathionylated at specific cysteine residues within the DNA-binding domain during oxidative stress (Velu et al., 2007), which may inhibit p53-dependent gene transcription, or may alter the affinity of p53 for particular gene promoters during oxidative stress conditions.

There are many other redox-regulated proteins in higher eukaryotes, and these are involved in a wide range of processes and pathways. Well-known redox-regulated proteins include PKA, whereby the formation of an intramolecular disulfide bond within the catalytic subunit of the enzyme promotes its inactivation (Humphries et al., 2002) and HIF, which is stabilised during hypoxia due to the redox-inhibition of PHD2 (although how ROS promote PHD2 inactivation is unclear) (Cash et al., 2007). However, identifying *all* redox-sensitive proteins, determining their relative sensitivities to cysteine oxidation, and integrating all the resulting responses together to predict overall cellular responses, remains a challenge. Interestingly, E1s and E2s facilitating ubiquitin/Ubl conjugation are also emerging as redox-sensitive proteins in eukaryotes.

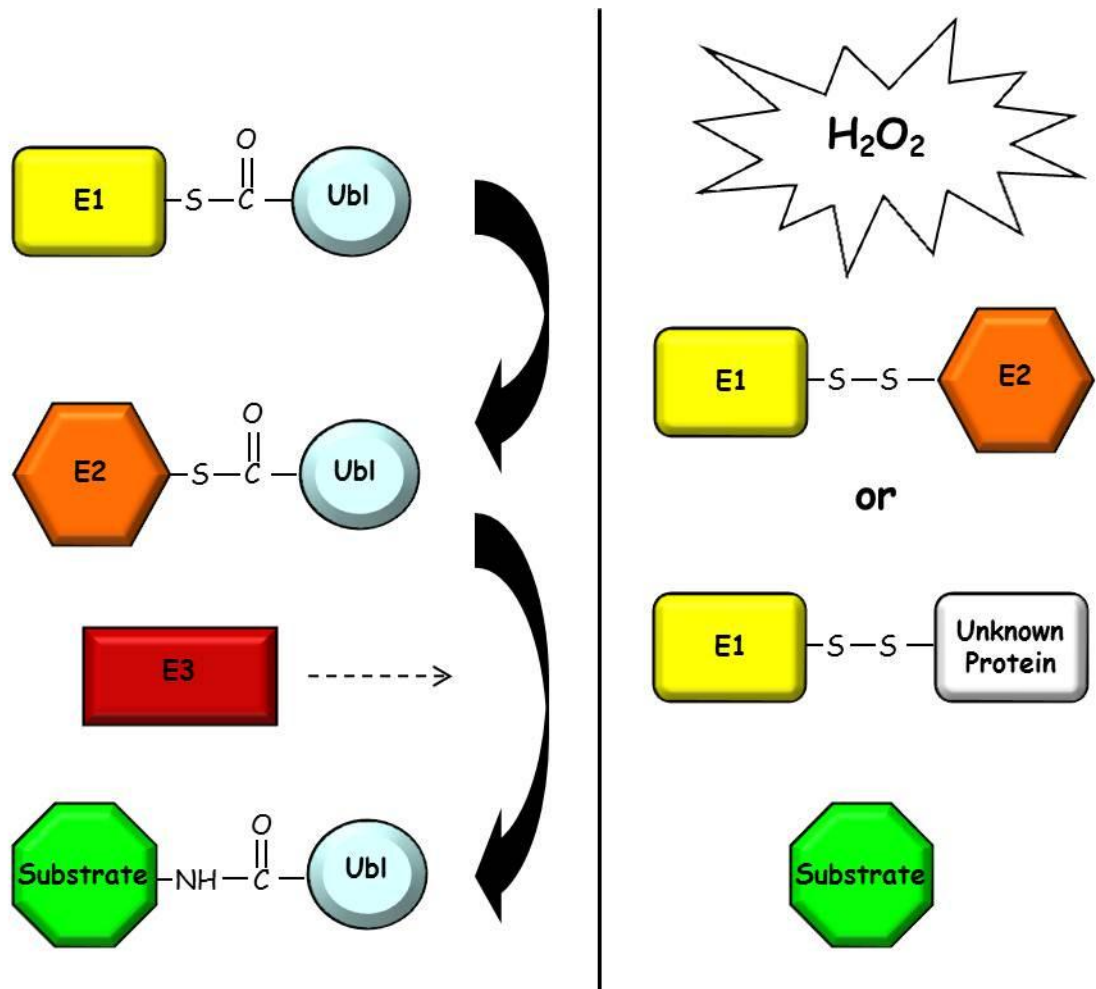
### **1.2.3. Redox regulation of ubiquitin/Ubl conjugation**

As described in Section 1.1, the conjugation of each ubiquitin/Ubl is facilitated by specific enzymes. E1s and E2s use catalytic cysteine residues to bind ubiquitin/Ubls and promote their conjugation to proteins. The oxidation of catalytic cysteines is an established regulatory mechanism by which enzymes such as PTPs can be inhibited. Given that the catalytic cysteines of E1s and E2s must be deprotonated to allow ubiquitin/Ubl thioester formation, and that these cysteines are likely to encounter distinct microenvironments during the catalytic cycle which may alter their relative pKa values, it is possible that H<sub>2</sub>O<sub>2</sub> can oxidise the catalytic cysteines of E1s and E2s to inhibit ubiquitin/Ubl conjugation. DUBs and ULPs are also candidates for redox regulation, as they utilise catalytic cysteines as part of a catalytic mechanism removing ubiquitin/Ubls from substrates. Recent studies in mammalian cells have shown that ubiquitin/Ubl conjugation can indeed be redox-regulated.

#### *1.2.3.1. Redox regulation of Ubl conjugation in mammals*

SUMOylation was the first ubiquitin/Ubl conjugation pathway found to be inhibited during mild oxidative stress in mammalian cells. It was observed that low concentrations of H<sub>2</sub>O<sub>2</sub> triggered the formation of an inhibitory disulfide bond between the catalytic cysteines of the E1, Uba2, and the E2, Ubc9 (**Fig. 1.8**) (Bossis and Melchior, 2006). This indicated that the catalytic cysteine of either Uba2 or Ubc9 (or both) is sensitive to oxidation by H<sub>2</sub>O<sub>2</sub>. The formation of an Uba2-Ubc9 disulfide complex prevented protein SUMOylation, as the catalytic cysteines of Uba2 and Ubc9 could not form thioesters with SUMO. In contrast, SENPs remained active, leading to the deSUMOylation of substrates that had been modified previously. Importantly, the inhibition of SUMOylation was only transient, as cellular reductants such as GSH could reverse Uba2-Ubc9 disulfide formation once redox homeostasis was restored. Furthermore, ubiquitination did not appear to be inhibited by H<sub>2</sub>O<sub>2</sub>. This demonstrated that the conjugation of a specific Ubl could be inhibited by H<sub>2</sub>O<sub>2</sub>. It was proposed that the decreased SUMOylation of transcription factors such as the AP-1 components c-Fos and c-Jun could increase the expression of target genes, including antioxidants, as part of an oxidative stress response (Bossis and Melchior, 2006). However, this was not investigated further. Thus, it remains unclear how inhibition of SUMOylation may contribute to oxidative stress resistance, or how other downstream processes may be affected.

Additionally, neddylation can be redox-regulated in human cells. The gut flora is comprised of many commensal bacteria, including non-pathogenic *E. coli*. The release of bacterial fermentation products such as butyrate somehow causes ROS generation in intestinal epithelial cells, resulting in oxidation of the Ubc12 catalytic cysteine residue and the sequestration of Ubc12 in higher M<sub>r</sub> disulfide complexes (**Fig. 1.8**) (Kumar et al., 2007; Kumar et al., 2009). It is unclear whether these complexes also include Uba3. However, the sequestration of Ubc12 would be sufficient to inhibit protein neddylation, as Ubc12 is the only E2 functioning in the neddylation pathway. By preventing neddylation of CRLs, the ubiquitination of many proteins would be significantly affected. Indeed, the inhibition of neddylation was shown to decrease NFκB-dependent transcription, as the ubiquitin-dependent proteolytic degradation of IκB was prevented (Kumar et al., 2007). This could be advantageous for bacteria, as inhibition of NFκB would be expected to decrease inflammation and immune responses in the gut. However, how redox regulation of neddylation affected oxidative stress responses in intestinal epithelial cells was not investigated. This would have been particularly



**Figure 1.8. Redox regulation of Ubl conjugation in mammalian cells.**

Oxidative stress can inhibit both SUMOylation and neddylation in mammalian cells. Low levels of  $\text{H}_2\text{O}_2$  trigger the formation of a disulfide bond between the catalytic cysteines of Uba2 and Ubc9, the E1 and E2 enzymes of the SUMOylation pathway. Uba2-Ubc9 complex formation prevents Uba2-SUMO and Ubc9-SUMO thioester formation, thus inhibiting global SUMOylation of proteins.  $\text{H}_2\text{O}_2$  can also oxidise the catalytic cysteine of the NEDD8-specific E2, Ubc12. This again causes inhibitory disulfide formation, although the NEDD8-specific E1, Uba3, has not been found to be involved in these complexes. Inhibition of Ubc12 blocks the neddylation of substrates including cullins. It is possible that other ubiquitin/Ubl conjugation pathways are redox-regulated in a similar fashion, both in mammals and lower eukaryotes.

interesting, as NF $\kappa$ B is known to regulate the expression of antioxidant genes in eukaryotes (Gius et al., 1999). It is possible that redox regulation of SUMOylation and neddylation is important for oxidative stress resistance, and may somehow influence key stress response pathways in cells.

As well as E1s and E2s, ULPs can be redox-regulated in mammalian cells. For example, the Atg8-specific protease, Atg4, is inhibited by oxidation of a cysteine residue near the catalytic site (Scherz-Shouval et al., 2007). According to the model proposed by Scherz-Shouval *et al.* (Scherz-Shouval and Elazar, 2007; Scherz-Shouval et al., 2007), this results in increased autophagic activity, as ROS generation by mitochondria may cause localised inhibition of Atg4, causing retention of Atg8-PE on autophagosomal membranes and promoting expansion of autophagosomes. Despite this, Atg4 would remain active elsewhere in the cell, allowing cleavage of precursor Atg8 to continue (**Fig. 1.4**). Redox regulation of Atg4 represents an important molecular mechanism for the induction of autophagy, and has been proposed to remove damaged mitochondria from cells to protect against oxidative stress (Scherz-Shouval and Elazar, 2007; Scherz-Shouval et al., 2007). Furthermore, inhibition of SENPs has been detected at high concentrations of H<sub>2</sub>O<sub>2</sub> (Bossis and Melchior, 2006), possibly explaining prior observations that SUMOylation is induced during severe oxidative stress (Zhou et al., 2004). SENPs are also known to form oligomers following oxidation, possibly to prevent hyperoxidation of catalytic cysteines (Xu et al., 2008). However, the high concentrations of H<sub>2</sub>O<sub>2</sub> required for SENP inhibition are unlikely to be physiologically relevant.

Although these findings are highly significant, many unanswered questions remained. Firstly, the consequences of inhibition of ubiquitin/Ubl conjugation on oxidative stress resistance and response mechanisms were not fully investigated. Secondly, redox regulation of ubiquitin/Ubl conjugation was only shown in mammalian cells. It was therefore unclear whether these regulatory mechanisms are species-specific or conserved. Furthermore, it was unclear why different enzymes show different degrees of redox-sensitivity. Recent research in our lab has addressed some of these issues. We have observed redox regulation of ubiquitin/Ubl conjugation in lower eukaryotes, and have found this to be important during responses to oxidative stress.

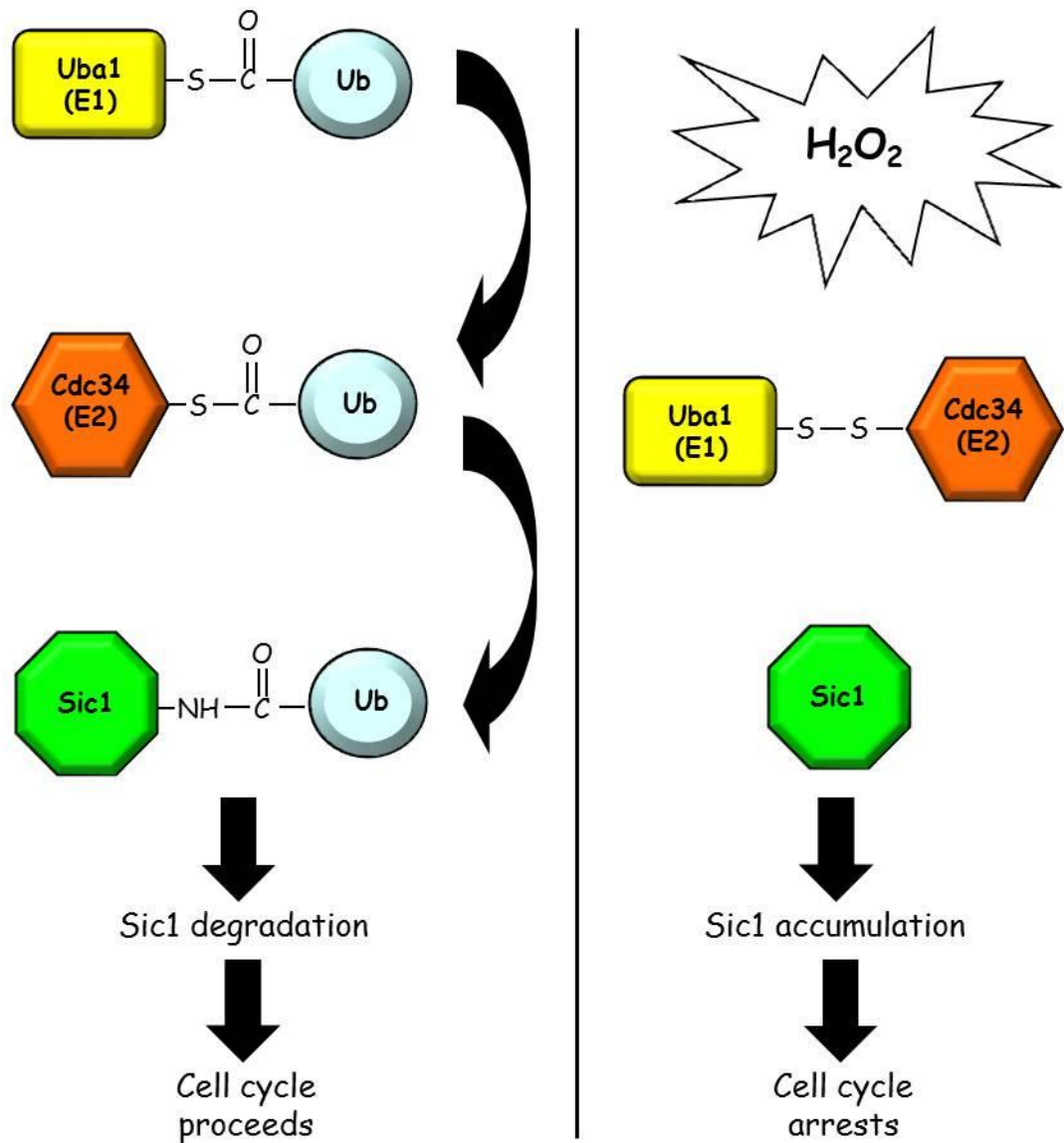
### 1.2.3.2. Ubiquitination of specific proteins is redox-regulated in lower eukaryotes

Previous investigations have shown that ubiquitination is generally inhibited during oxidative stress. Levels of ubiquitinated proteins and E1/E2-ubiquitin thioesters are decreased during oxidative stress, possibly due to glutathionylation of E1s and E2s at their catalytic cysteine residues (Jahngen-Hodge et al., 1997; Obin et al., 1998). However, there is also evidence that these enzymes may not be redox-regulated. Many ubiquitin-specific E2s appear to have catalytic cysteines with high pKa values (Tolbert et al., 2005), which would likely prevent their deprotonation and oxidation. Furthermore, while SUMOylation is inhibited during oxidative stress in mammalian cells, there was no detectable effect on ubiquitination (Bossis and Melchior, 2006). Finally, although cysteine oxidation leads to the formation of a complex involving a ubiquitin-specific E2, UbcM2, and the antioxidant transcription factor Nrf2, and somehow increases the activity of Nrf2 (Plafker et al., 2010), the catalytic cysteine of UbcM2 was not oxidised.

Despite this, we have observed the formation of an inhibitory disulfide complex involving Uba1 and a downstream E2, Cdc34, as a result of catalytic cysteine oxidation in *S. cerevisiae* (unpublished data). This causes the stabilisation of the Cdk1, Sic1, leading to a transient cell cycle arrest during G1 phase. The resulting delay in S phase onset would allow the cell to restore redox homeostasis and thus prevent oxidative damage to chromosomes prior to DNA replication. Significantly, Uba1 does not appear to form higher  $M_r$  complexes with any other E2s during oxidative stress, allowing ubiquitination of other substrates to continue unaffected (**Fig. 1.9**). This is in contrast to redox regulation of SUMOylation and neddylation in mammalian cells, which affects *all* SUMO and NEDD8 substrates. Furthermore, it is possible that the ubiquitination of specific substrates is also inhibited by oxidative stress in other organisms, but is difficult to detect as the ubiquitination of hundreds of other proteins continues unaffected. These results show that redox regulation of ubiquitin/Ubl conjugation occurs in higher and lower eukaryotes and can play important roles in oxidative stress resistance, and that certain enzymes are more sensitive to cysteine oxidation than others.

Clearly, redox regulation of protein activity is an important mechanism influencing many cellular proteins and pathways under homeostatic conditions, and also in response to oxidative stress. The cell cycle delay resulting from Uba1-Cdc34 disulfide formation





**Figure 1.9. Ubiquitination and degradation of Sic1 is redox-regulated in *S. cerevisiae*.**

Oxidative stress induced by  $H_2O_2$  or diamide triggers the formation of a disulfide bond between the catalytic cysteines of Uba1 and a specific E2 enzyme, Cdc34. This prevents the Cdc34-dependent ubiquitination and degradation of a Cdk inhibitor, Sic1, leading to cell cycle arrest in G1 phase until redox homeostasis is restored. No other E2s appear to form disulfides with Uba1, leaving the vast majority of ubiquitination events unaffected. Our data demonstrates that ubiquitin/Ubl conjugation is redox regulated in both higher and lower organisms, and that only certain enzymes have redox-sensitive catalytic cysteine residues.

and Sic1 stabilisation is an example of this. However, it is possible that other E1s and E2s are redox-sensitive, and may also play roles in controlling stress resistance. Oxidative stress response mechanisms are particularly well-characterised in model organisms such as *S. pombe*, and are highly conserved from yeast to human. Thus, by studying model organisms such as *S. cerevisiae* and *S. pombe*, we can gain an insight into biochemical pathways and regulatory mechanisms also functioning in mammalian cells. Redox regulation of ubiquitin/Ubl conjugation pathways may be a conserved mechanism through which oxidative stress responses may be controlled. In this thesis, the redox-sensitivity of enzymes facilitating ubiquitin/Ubl conjugation will be investigated in *S. pombe*. In evolutionary terms, *S. cerevisiae* and *S. pombe* are as far removed from each other as from humans. Thus, by comparing our knowledge of humans and *S. cerevisiae* with our knowledge of *S. pombe*, the extent to which regulatory mechanisms are conserved in eukaryotes can be determined. It is possible that redox regulation of certain E1s and E2s, such as Uba1 and Cdc34, is conserved, and is a highly-important response mechanism in eukaryotic cells. Our investigations will also shed light on ubiquitin/Ubl conjugation pathways in fission yeast, which are relatively uncharacterised in comparison to those of *S. cerevisiae* or mammalian cells. Furthermore, how ubiquitin/Ubl conjugation pathways (and any redox regulation of these pathways) may affect oxidative stress response mechanisms in *S. pombe* will be investigated. These pathways are very well-characterised, leading us to select fission yeast as a model organism for our studies. The oxidative stress response mechanisms of *S. pombe* are outlined in the next section.

### **1.3. Oxidative Stress Responses in *S. pombe*.**

Oxidative stress response mechanisms allow *S. pombe* to mount distinct transcriptional responses according to the level of stress (Quinn et al., 2002). Changes in the transcriptional response with time and in response to different concentrations of H<sub>2</sub>O<sub>2</sub> have also been demonstrated by microarray studies (Chen et al., 2008a). The Pap1 transcription factor is important for responses to mild oxidative stress. However, responses to higher concentrations of H<sub>2</sub>O<sub>2</sub> are largely controlled by the SAPK, Sty1. Furthermore, the sole 2-Cys Prx in fission yeast, Tpx1, is involved in controlling the activation of both Pap1 and Sty1. Finally, a second SAPK, Pmk1, also appears to have roles in the oxidative stress responses of *S. pombe*.

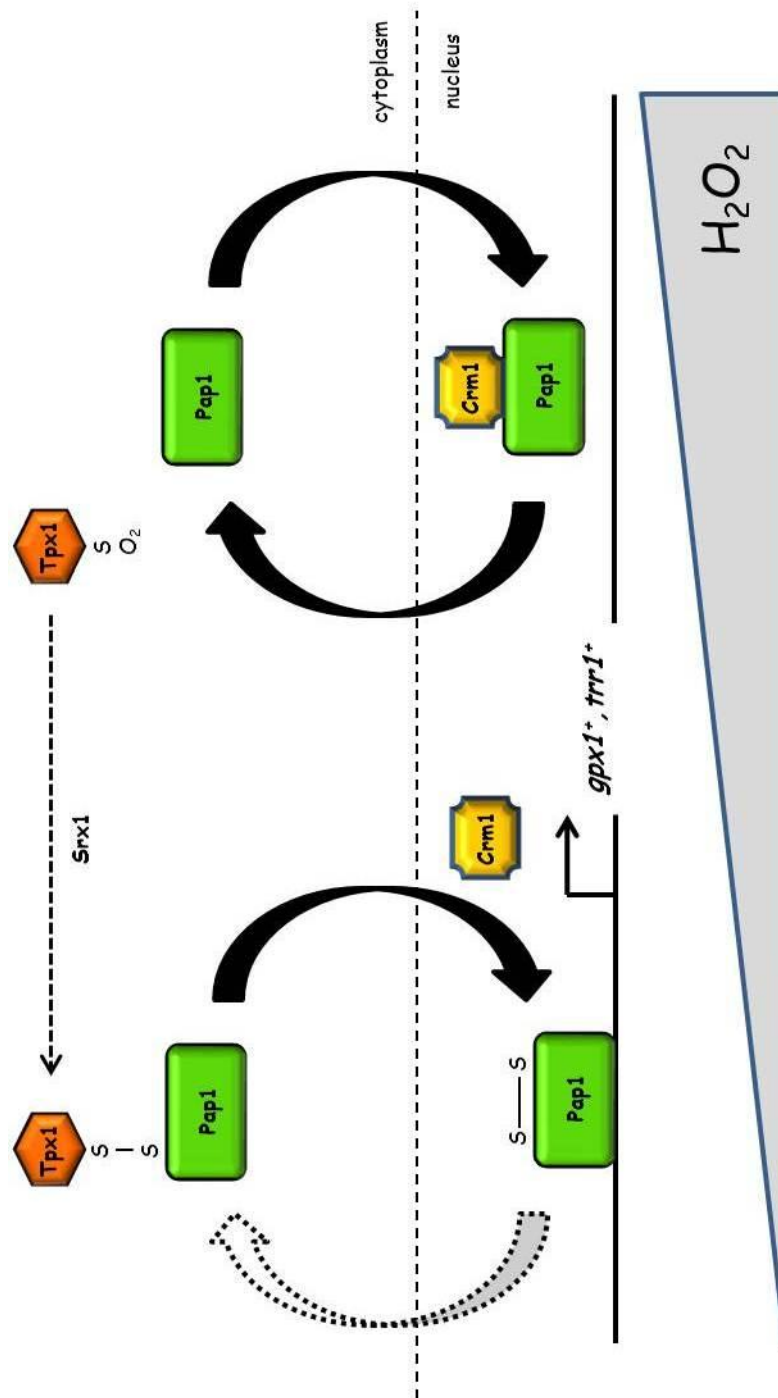
#### **1.3.1. Regulation of the transcription factor Pap1 by the 2-Cys Prx, Tpx1**

The *S. pombe* bZIP transcription factor Pap1 was originally identified by homology to c-Jun, a component of mammalian AP-1 transcription factors activated by JNK in response to stress (Toda et al., 1991). In unstressed cells, Pap1 cycles between the cytoplasm and the nucleus, but accumulates in the nucleus following oxidative stress, thus activating the transcription of numerous antioxidant genes (Madrid et al., 2004; Toone et al., 1998). Interestingly, Pap1 accumulates in the nucleus faster at lower concentrations of H<sub>2</sub>O<sub>2</sub> (Quinn et al., 2002), and this is reflected by a delay in Pap1-dependent gene expression at high concentrations of H<sub>2</sub>O<sub>2</sub> (Quinn et al., 2002). Significantly, a NES is located within a CRD at the C-terminus of Pap1 (c-CRD), allowing the Crm1 export factor to facilitate Pap1 nuclear export (Kudo et al., 1999). At low concentrations of H<sub>2</sub>O<sub>2</sub>, cysteine residues located within the c-CRD and a second CRD near the N-terminus (n-CRD) form an intramolecular disulfide, thus masking the NES and preventing the nuclear export of Pap1 (Castillo et al., 2002; Vivancos et al., 2004). Furthermore, Pap1 regulates the expression of genes encoding components of the

thioredoxin pathway, which may represent a negative feedback mechanism by which intramolecular disulfides within Pap1 can be reversed (Veal et al., 2007). Finally, it has recently been demonstrated that active, nuclear Pap1 is targeted for degradation by the ubiquitin ligase Ubr1 (Kitamura et al., 2011). This may allow *S. pombe* to rapidly halt Pap1-dependent gene transcription once redox homeostasis has been restored. The mechanism of Pap1 activation is very similar to that of the *S. cerevisiae* homologue, Yap1, which also regulates oxidative stress responses (Fernandes et al., 1997; Kuge et al., 1997) and accumulates in the nucleus following cysteine oxidation and intramolecular disulfide formation (Delaunay et al., 2000). Indeed, AP-1-like transcription factors are regulated at the level of nuclear localisation in response to oxidative stress in many model organisms (Ikner and Shiozaki, 2005), emphasising the conservation of these regulatory mechanisms and their importance in protecting cells against oxidative stress.

Interestingly, Tpx1 is required for the oxidation of Pap1 in response to H<sub>2</sub>O<sub>2</sub>. Moreover, hyperoxidation of the peroxidatic cysteine of Tpx1 at higher levels of H<sub>2</sub>O<sub>2</sub> prevents Pap1 oxidation until Tpx1 can be reactivated by a Srx enzyme (**Fig. 1.10**) (Bozonet et al., 2005; Kanki and Klionsky, 2008; Veal et al., 2004; Vivancos et al., 2005; Vivancos et al., 2006). This led to the proposal that Tpx1 acts as a ‘redox transducer’ and a ‘molecular switch’ that allows fission yeast to mount distinct responses to different levels of H<sub>2</sub>O<sub>2</sub> (Bozonet et al., 2005; Veal et al., 2007). Significantly, the *S. cerevisiae* antioxidant enzyme Gpx3 also acts as a redox transducer to facilitate the oxidation and nuclear accumulation of Yap1 (Delaunay et al., 2002), and a 2-Cys Prx, Tsa1, can fulfil this role in *S. cerevisiae* lacking the Ybp1 protein (Okazaki et al., 2005). Furthermore, recent evidence suggests that Ahp1 functions as a redox transducer to promote the oxidation and activation of a second budding yeast transcription factor, Yap2 (Iwai et al., 2010). Indeed, recent research in budding yeast has emphasised the role of thiol peroxidases as redox transducers with widespread effects on gene expression (Fomenko et al., 2011; Gutscher et al., 2009). Thus, the redox regulation of transcription factor activity via antioxidant enzymes appears to be a conserved mechanism in eukaryotes, and is exemplified by the Tpx1-mediated nuclear accumulation of Pap1 in *S. pombe*.

### 1.3.2. The Sty1 pathway



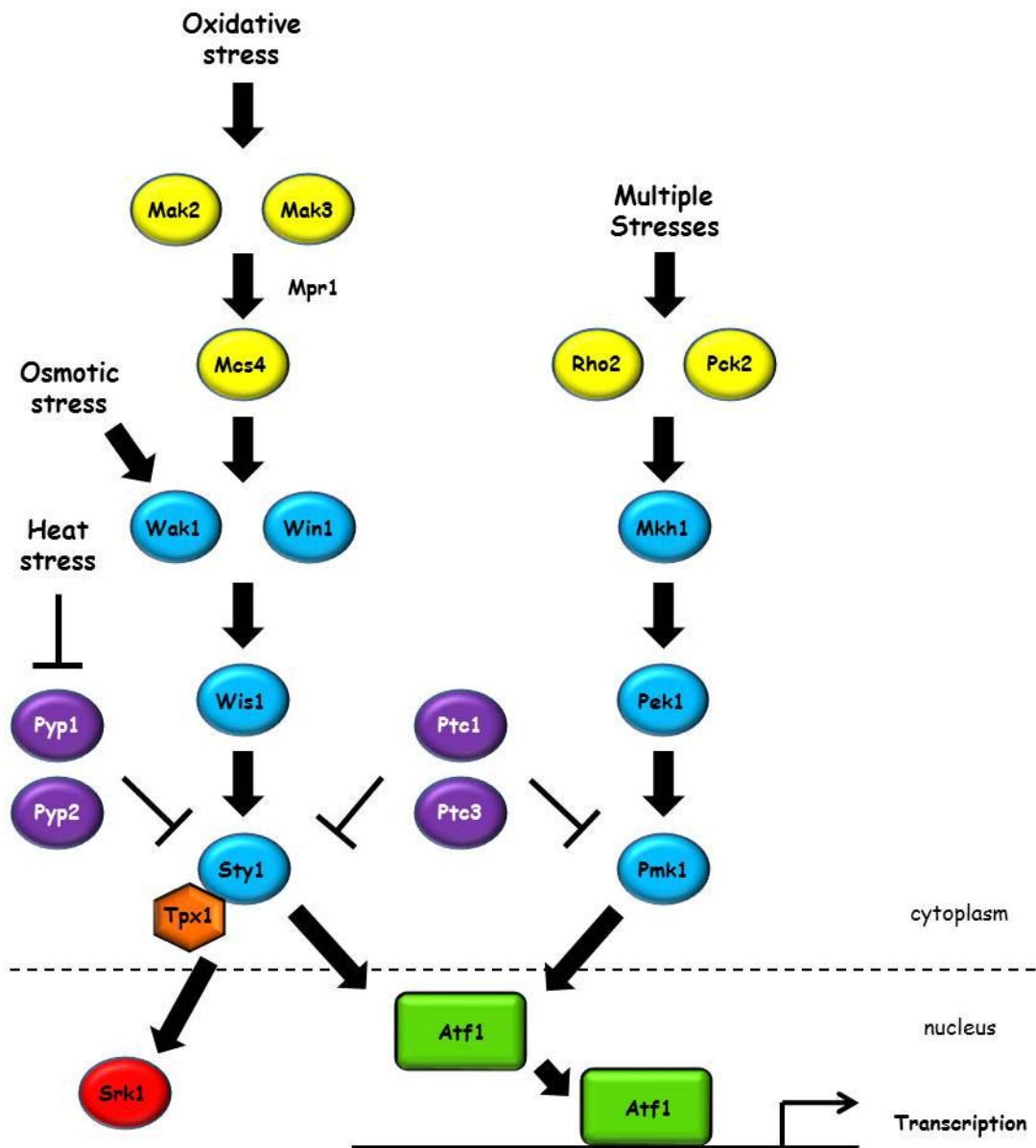
**Figure 1.10. The Pap1 transcription factor accumulates in the nucleus in response to mild oxidative stress, but this is delayed at higher concentrations of  $H_2O_2$ .**

The bZIP transcription factor Pap1 cycles between the cytoplasm and the nucleus under homeostatic conditions. Mild oxidative stress (e.g. 0.2mM  $H_2O_2$ ) causes the formation of an intramolecular disulfide between two CRDs of the protein, via the redox transducer Tpx1. Disulfide formation shields the Pap1 nuclear export signal (NES) from the export adaptor Crm1, causing Pap1 to accumulate in the nucleus where it activates a transcriptional response, allowing adaptation to oxidative stress conditions. In contrast, higher concentrations of  $H_2O_2$  (e.g. 1mM) cause hyperoxidation of Tpx1, inhibiting its function as a redox transducer, and preventing Pap1 from accumulating in the nucleus. Other oxidative stress responses (mediated largely by a second transcription factor, Atf1) decrease intracellular  $H_2O_2$  concentrations and induce the expression of Srx1 to reactivate Tpx1, which eventually promotes Pap1 nuclear accumulation as the cellular oxidative stress response proceeds.

The Sty1 SAPK is homologous to the mammalian p38/JNK SAPKs, and regulates responses to a range of stresses, including oxidative stress, osmotic stress, heat shock and nutrient deprivation (Degols et al., 1996; Kato et al., 1996; Millar et al., 1995; Shiozaki and Russell, 1995; Toone et al., 1998). Sty1 forms part of a MAPK cascade, involving the MAPKKKs Wak1 (Samejima et al., 1997; Shieh et al., 1997; Shiozaki et al., 1997) and Win1 (Shieh et al., 1998), and the MAPKK Wis1 (Kato et al., 1996; Millar et al., 1995; Shiozaki and Russell, 1995) (**Fig. 1.11**). This cascade is conserved in other model organisms, such as *Saccharomyces cerevisiae* (Ikner and Shiozaki, 2005). However, the budding yeast SAPK, Hog1, is only activated by osmotic stress (Brewster et al., 1993; Schuller et al., 1994). This contrasts with SAPK cascades in *S. pombe* and humans (Ikner and Shiozaki, 2005), indicating that studies of oxidative stress responses in *S. pombe* could provide a better insight into stress response mechanisms in human cells.

The H<sub>2</sub>O<sub>2</sub>-induced activation of Sty1 involves the MAPK cascade and a two-component phosphorelay module, which is involved in sensing H<sub>2</sub>O<sub>2</sub> levels upstream of the MAPKKKs (Buck et al., 2001). The histidine kinases Mak2 and Mak3 are activated by H<sub>2</sub>O<sub>2</sub> – possibly via stress-induced conformational changes – and subsequently activate the response regulator Mcs4, which then triggers the MAPK cascade (Buck et al., 2001) (**Fig. 1.11**). In addition, Tpx1 is required for the optimal activation of Sty1 in response to H<sub>2</sub>O<sub>2</sub> (Veal et al., 2004). Increased concentrations of H<sub>2</sub>O<sub>2</sub> cause the formation of a disulfide bond between Sty1 and Tpx1, possibly to prevent the inhibitory oxidation of a cysteine residue important for Sty1 activity (Veal et al., 2004). Significantly, although Sty1 is activated by a range of stresses, the mechanisms of activation vary. Sty1 activation is mediated by inhibition of the Sty1-specific tyrosine phosphatase Pyp1 during heat shock (Nguyen and Shiozaki, 1999; Shiozaki et al., 1998), thus bypassing the MAPK cascade. This may also be the case during arsenite exposure (Rodriguez-Gabriel and Russell, 2005). The different mechanisms of Sty1 activation have been reviewed elsewhere (Zhou et al., 2010).

A key downstream target of Sty1 is the bZIP transcription factor Atf1, which is related to mammalian ATF-2. The phosphorylation of Atf1 by Sty1 is very similar to the phosphorylation of ATF-2 by mammalian SAPKs in response to stress (Gupta et al., 1995; Livingstone et al., 1995), again demonstrating that *S. pombe* stress response mechanisms could be representative of those in higher organisms. Atf1 forms a



**Figure 1.11. The Sty1 and Pmk1 SAPK cascades in *S. pombe*.**

The histidine kinases Mak2/3 and the response regulator Mcs4 form a two-component signalling module that becomes activated in response to  $H_2O_2$ . An activation signal is then relayed to the SAPK, Sty1. Sty1 is activated as part of a MAPK cascade involving the MAPKK Wis1 and the MAPKKKs Wak1 and Win1. Furthermore, a 2-Cys Prx, Tpx1, forms a disulfide bond with Sty1 to promote activation of Sty1 in response to  $H_2O_2$ . Importantly, Sty1 is activated in response to many other stresses, including osmotic stress. Moreover, a Sty1 phosphatase, Pyp1, is inhibited during temperature stress, indicating that there are multiple mechanisms of Sty1 activation. Activated Sty1 then translocates to the nucleus and activates the transcription factor Atf1, which promotes the expression of a range of genes involved in stress responses. Sty1 can also activate Srk1, a kinase with roles in cell cycle regulation. A second SAPK, Pmk1, is activated by a MAPK cascade involving the MAPKK Pek1 and the MAPKKK Mkh1. Pmk1 is also important for stress resistance and can activate Atf1-dependent gene transcription. Both Sty1 and Pmk1 can be inactivated by the Ptc1 and Ptc3 phosphatases.

heterodimer with Pcr1 (Takeda et al., 1995; Watanabe and Yamamoto, 1996; Wilkinson et al., 1996), although recent evidence suggests that Atf1 may also be able to regulate transcription independently of Pcr1 (Sanso et al., 2008). Atf1 is phosphorylated and activated by Sty1, and increases the expression of numerous antioxidant genes (Chen et al., 2008a) including *ctt1*<sup>+</sup>, encoding catalase (Wilkinson et al., 1996), and *gpx1*<sup>+</sup>, encoding glutathione peroxidase (Yamada et al., 1999). Atf1 also activates *pyp2*<sup>+</sup>, encoding the Sty1-specific tyrosine phosphatase Pyp2, as part of a negative feedback mechanism (Wilkinson et al., 1996). Atf1 phosphorylation increases as the H<sub>2</sub>O<sub>2</sub> concentration increases (Quinn et al., 2002), indicating that Atf1 is important for survival during severe oxidative stress. Phosphorylation of Atf1 has also been shown to prevent proteasomal degradation of the protein (Lawrence et al., 2009). In addition to post-translational modification, Atf1 activity may be controlled at the mRNA level during oxidative stress via Sty1-dependent (Day and Veal, 2010; Rodriguez-Gabriel et al., 2003) and -independent (Rodriguez-Gabriel et al., 2006) mechanisms.

Interestingly, Sty1-dependent responses to some stresses, such as arsenite exposure, do not require Atf1 (Rodriguez-Gabriel and Russell, 2005). Furthermore, some phenotypes of the  $\Delta$ *sty1* strain, including a cell cycle delay resulting in cell elongation (Millar et al., 1995; Shiozaki and Russell, 1995), are not observed in  $\Delta$ *atf1* cells (Wilkinson et al., 1996), indicating that there are other downstream effectors of Sty1. Indeed, the protein kinase Srk1 is phosphorylated and activated by Sty1 (Smith et al., 2002). Following activation, Srk1 inhibits the Cdc25 phosphatase to promote cell cycle arrest at the G2/M phase boundary in response to stress (Lopez-Aviles et al., 2005; Smith et al., 2002). Srk1 is also involved in the control of nitrogen starvation-induced arrest in G1 phase (Smith et al., 2002). Significantly, Srk1 phosphorylation decreases the stability of the kinase, resulting in the rapid degradation of active Srk1 when Sty1 activity is decreased (Lopez-Aviles et al., 2008). Furthermore, Sty1 phosphorylates the polo kinase Plo1 to promote Plo1 localisation to spindle pole bodies, which is required for efficient mitosis (Petersen and Hagan, 2005). Aberrant Plo1 localisation may partly explain why  $\Delta$ *sty1* cells show delays in mitotic initiation, resulting in cell elongation. The protein kinase, Cmk2, is another Sty1 substrate, and contributes to oxidative stress responses (Sanchez-Piris et al., 2002). Sty1 is also involved in the control of DNA damage responses (Alao and Sunnerhagen, 2008), the regulation of translation during stress (Asp et al., 2008; Berlanga et al., 2010), and lifespan extension via calorie restriction (Zuin et al., 2010).



As outlined in Section 1.3.1, the Pap1 pathway is largely required for cellular adaptation to mild H<sub>2</sub>O<sub>2</sub> stress, while the Sty1/Atf1 pathway is required for survival at high concentrations of H<sub>2</sub>O<sub>2</sub> (Quinn et al., 2002; Vivancos et al., 2006); a model supported by microarray data showing that Pap1-dependent gene expression is highest at low H<sub>2</sub>O<sub>2</sub>, while Atf1-dependent gene expression increases at high H<sub>2</sub>O<sub>2</sub> (Chen et al., 2008a). However, Pap1 eventually accumulates in the nucleus and regulates gene expression during more severe stress, indicating that Pap1-dependent gene expression is required later in the response (Quinn et al., 2002). In a key example of cross-talk between the different pathways, Atf1 regulates the expression of *srx1*<sup>+</sup>, encoding sulfiredoxin, which is able to reactivate hyperoxidised Tpx1 and allow Pap1 activation following initial activation of the Sty1/Atf1 pathway (Bozonet et al., 2005; Vivancos et al., 2005). Sty1/Atf1-dependent oxidative stress responses may also reduce intracellular H<sub>2</sub>O<sub>2</sub> concentrations to a level at which Tpx1 is not hyperoxidised, and is able to activate Pap1.

Clearly, the Tpx1-Pap1 and Sty1/Atf1 pathways represent a highly sophisticated oxidative stress response system, allowing *S. pombe* to tailor transcriptional responses in response to different levels of oxidant. The messenger function of H<sub>2</sub>O<sub>2</sub> is critical for these responses. However, it has become clear that Pmk1 is also involved in oxidative stress responses in fission yeast, adding further complexity to the system.

### **1.3.3. The Pmk1 pathway**

Pmk1 was originally identified as a MAPK with roles in maintaining cell wall integrity, morphology and septa formation during cytokinesis in *S. pombe* (Toda et al., 1996; Zaitsevskaya-Carter and Cooper, 1997). Pmk1 phosphorylation and activation appears to be linked to the cell cycle, peaking during cytokinesis (Madrid et al., 2007). Additionally, Pmk1 was shown to be activated in response to osmotic and temperature stresses and nutrient deprivation (Zaitsevskaya-Carter and Cooper, 1997). As outlined above, Sty1 is also activated by these stress conditions. However, it was proposed that

Pmk1 controls morphological and cytokinetic changes required during stress, in contrast to Sty1, which regulates transcriptional responses to combat stress directly (Zaitsevskaia-Carter and Cooper, 1997). Further investigations identified the MAPKKK Mkh1 (Sengar et al., 1997) and the MAPKK Pek1 (Loewith et al., 2000; Sugiura et al., 1999) functioning upstream of Pmk1 (**Fig. 1.11**). More recently, it has been shown that the small GTPase Rho2 and the PKC homologue Pck2 may function upstream of Mkh1 to regulate Pmk1 activity during the cell cycle and in response to osmotic stress (**Fig. 1.11**) (Barba et al., 2008).

Recent studies demonstrated that Pmk1 is activated when cells are exposed to H<sub>2</sub>O<sub>2</sub> (Madrid et al., 2006). However, in contrast to Sty1, Pmk1 is also activated when cells are exposed to the thiol-oxidising agent diamide and the alkyl hydroperoxide *t*-BOOH. Furthermore,  $\Delta pmk1$  cells display increased sensitivity to diamide and *t*-BOOH, but not H<sub>2</sub>O<sub>2</sub>, while  $\Delta sty1$  cells display increased sensitivity to H<sub>2</sub>O<sub>2</sub> but not diamide and *t*-BOOH (Chen et al., 2008a; Madrid et al., 2006). Pmk1 is also required for resistance to ROS generated following inhibition of mETC complex I (Wang et al., 2010). Clearly, Pmk1 influences oxidative stress responses in *S. pombe*, and Pmk1 may be particularly important when Sty1 is unresponsive to a particular stress (and vice-versa).

Interestingly, Atf1 appears to be a downstream target of both Sty1 and Pmk1 (**Fig. 1.11**) (Takada et al., 2007). Indeed, microarray analysis suggests that Pmk1 regulates antioxidant gene expression during *t*-BOOH stress in an Atf1-dependent manner (Chen et al., 2008a), although other downstream substrates of Pmk1 could also be important for oxidative stress resistance. There is also evidence of cross-talk between the two SAPK pathways, as Sty1 activation leads to inactivation of Pmk1 via the Atf1-dependent upregulation of Pmk1 phosphatases (Madrid et al., 2007; Madrid et al., 2006). Significantly, it is unclear whether Atf1 is phosphorylated on the same sites by Sty1 and Pmk1 (Takada et al., 2007). It is possible that phosphorylation of Atf1 on different sites by different MAPKs influences the genes regulated by Atf1 under certain conditions, allowing the cell to alter transcriptional responses depending on the stress agent. However, if Atf1 is phosphorylated at the same sites by Sty1 and Pmk1, there must be another mechanism determining the genes regulated by Atf1 following Sty1 or Pmk1 activation.

## 1.4. Summary

Many ubiquitin/Ubl conjugation pathways are highly-conserved in eukaryotes, and can influence many important cellular processes. Previous investigations have established that ubiquitin/Ubl conjugation can be redox-regulated in lower and higher eukaryotes. Moreover, our data suggests that redox regulation of Sic1 ubiquitination could be important for the survival of *S. cerevisiae* during oxidative stress. However, while redox regulation of protein activity is crucial for the control of oxidative stress responses, it is largely unclear how redox regulation of ubiquitin/Ubl conjugation contributes to oxidative stress resistance. It is also unclear why some E1s and E2s are redox-regulated and others are not.

Ubiquitin/Ubl conjugation pathways are relatively uncharacterised in *S. pombe*, in contrast to those of *S. cerevisiae* and mammalian cells. However, fission yeast oxidative stress response mechanisms are well-characterised, and yeast has proven to be an excellent model for the study of stress responses in eukaryotes. Given that redox-sensitive proteins in lower organisms are invariably involved in oxidative stress responses, it is likely that any redox regulation of ubiquitin/Ubl conjugation will impact on oxidative stress responses in *S. pombe*. In this thesis, redox regulation of ubiquitin/Ubl conjugation will be investigated in *S. pombe*. The importance of ubiquitin/Ubl conjugation pathways in oxidative stress resistance in fission yeast will also be investigated, and related to any redox regulation of the enzymes involved. Finally, the structural basis for redox regulation of specific E1s and E2s will be examined, which may allow us to predict which enzymes are the most redox-sensitive.

## 1.5. Aims and Objectives

Ubiquitin/Ubl conjugation pathways are highly conserved in eukaryotes, and regulate a range of important processes. Recent investigations have demonstrated redox regulation of specific ubiquitin/Ubl conjugation pathways in both higher organisms and the model yeast, *S. cerevisiae*. However, how this redox regulation may impact on responses to oxidative stress is largely unclear. Moreover, while pathways such as urmylation and autophagic Ubl conjugation are important for resistance to oxidative stress in certain organisms, it is unclear whether these pathways have conserved roles in stress resistance in eukaryotes. Hence, the aim of this thesis is to investigate redox regulation of ubiquitin/Ubl conjugation in the fission yeast, *S. pombe*, determine the importance of these pathways in responses to oxidative stress, and to investigate why some pathways include redox-sensitive enzymes, while other pathways are unaffected by ROS such as H<sub>2</sub>O<sub>2</sub>.

Specific objectives include:

- Investigate redox regulation of ubiquitination and neddylation in *S. pombe*, and whether this is important for resistance to oxidative stress.
- Determine whether urmylation has conserved roles in stress resistance in eukaryotes, and whether this pathway is redox-regulated.
- Investigate autophagic Ubl conjugation and its relevance for oxidative stress responses in *S. pombe*, and the possible redox regulation of the process.



## References

- Adams, D.O., Johnson, W.J., and Marino, P.A. (1982). Mechanisms of target recognition and destruction in macrophage-mediated tumor cytotoxicity. *Federation proceedings* 41, 2212-2221.
- al-Khodairy, F., Enoch, T., Hagan, I.M., and Carr, A.M. (1995). The *Schizosaccharomyces pombe* hus5 gene encodes a ubiquitin conjugating enzyme required for normal mitosis. *Journal of cell science* 108 ( Pt 2), 475-486.
- Alao, J.P., and Sunnerhagen, P. (2008). Rad3 and Sty1 function in *Schizosaccharomyces pombe*: an integrated response to DNA damage and environmental stress? *Molecular microbiology* 68, 246-254.
- Ankar, J., and Sistonen, L. (2007). SUMO: getting it on. *Biochemical Society transactions* 35, 1409-1413.
- Arico, S., Petiot, A., Bauvy, C., Dubbelhuis, P.F., Meijer, A.J., Codogno, P., and Ogier-Denis, E. (2001). The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. *The Journal of biological chemistry* 276, 35243-35246.
- Ashraf, S.S., Sochacka, E., Cain, R., Guenther, R., Malkiewicz, A., and Agris, P.F. (1999). Single atom modification (O $\rightarrow$ S) of tRNA confers ribosome binding. *RNA* 5, 188-194.
- Asp, E., Nilsson, D., and Sunnerhagen, P. (2008). Fission yeast mitogen-activated protein kinase Sty1 interacts with translation factors. *Eukaryotic cell* 7, 328-338.
- Azad, M.B., Chen, Y., and Gibson, S.B. (2009). Regulation of autophagy by reactive oxygen species (ROS): implications for cancer progression and treatment. *Antioxidants & redox signaling* 11, 777-790.
- Bae, Y.S., Kang, S.W., Seo, M.S., Baines, I.C., Tekle, E., Chock, P.B., and Rhee, S.G. (1997). Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. *The Journal of biological chemistry* 272, 217-221.
- Bae, Y.S., Sung, J.Y., Kim, O.S., Kim, Y.J., Hur, K.C., Kazlauskas, A., and Rhee, S.G. (2000). Platelet-derived growth factor-induced H<sub>2</sub>O<sub>2</sub> production requires the activation of phosphatidylinositol 3-kinase. *The Journal of biological chemistry* 275, 10527-10531.
- Balaban, R.S., Nemoto, S., and Finkel, T. (2005). Mitochondria, oxidants, and aging. *Cell* 120, 483-495.
- Barba, G., Soto, T., Madrid, M., Nunez, A., Vicente, J., Gacto, M., and Cansado, J. (2008). Activation of the cell integrity pathway is channelled through diverse signalling elements in fission yeast. *Cellular signalling* 20, 748-757.
- Barford, D. (2004). The role of cysteine residues as redox-sensitive regulatory switches. *Current opinion in structural biology* 14, 679-686.
- Barrett, W.C., DeGnore, J.P., Konig, S., Fales, H.M., Keng, Y.F., Zhang, Z.Y., Yim, M.B., and Chock, P.B. (1999). Regulation of PTP1B via glutathionylation of the active site cysteine 215. *Biochemistry* 38, 6699-6705.
- Bayer, P., Arndt, A., Metzger, S., Mahajan, R., Melchior, F., Jaenicke, R., and Becker, J. (1998). Structure determination of the small ubiquitin-related modifier SUMO-1. *Journal of molecular biology* 280, 275-286.
- Beckman, K.B., and Ames, B.N. (1997). Oxidative decay of DNA. *The Journal of biological chemistry* 272, 19633-19636.
- Bedford, L., Lowe, J., Dick, L.R., Mayer, R.J., and Brownell, J.E. (2011). Ubiquitin-like protein conjugation and the ubiquitin-proteasome system as drug targets. *Nature reviews Drug discovery* 10, 29-46.

Bensaad, K., Cheung, E.C., and Vousden, K.H. (2009). Modulation of intracellular ROS levels by TIGAR controls autophagy. *The EMBO journal* 28, 3015-3026.

Berlangua, J.J., Rivero, D., Martin, R., Herrero, S., Moreno, S., and de Haro, C. (2010). Role of mitogen-activated protein kinase Sty1 in regulation of eukaryotic initiation factor 2 $\alpha$  kinases in response to environmental stress in *Schizosaccharomyces pombe*. *Eukaryotic cell* 9, 194-207.

Berlett, B.S., and Stadtman, E.R. (1997). Protein oxidation in aging, disease, and oxidative stress. *The Journal of biological chemistry* 272, 20313-20316.

Biteau, B., Labarre, J., and Toledano, M.B. (2003). ATP-dependent reduction of cysteine-sulphinic acid by *S. cerevisiae* sulphiredoxin. *Nature* 425, 980-984.

Bjork, G.R., Huang, B., Persson, O.P., and Bystrom, A.S. (2007). A conserved modified wobble nucleoside (mcm5s2U) in lysyl-tRNA is required for viability in yeast. *RNA* 13, 1245-1255.

Bjorkoy, G., Lamark, T., Brech, A., Outzen, H., Perander, M., Overvatn, A., Stenmark, H., and Johansen, T. (2005). p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *The Journal of cell biology* 171, 603-614.

Bossis, G., Malnou, C.E., Farras, R., Andermarcher, E., Hipkind, R., Rodriguez, M., Schmidt, D., Muller, S., Jariel-Encontre, I., and Piechaczyk, M. (2005). Down-regulation of c-Fos/c-Jun AP-1 dimer activity by sumoylation. *Molecular and cellular biology* 25, 6964-6979.

Bossis, G., and Melchior, F. (2006). Regulation of SUMOylation by reversible oxidation of SUMO conjugating enzymes. *Molecular cell* 21, 349-357.

Bosu, D.R., and Kipreos, E.T. (2008). Cullin-RING ubiquitin ligases: global regulation and activation cycles. *Cell division* 3, 7.

Boveris, A., and Chance, B. (1973). The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *The Biochemical journal* 134, 707-716.

Bozonet, S.M., Findlay, V.J., Day, A.M., Cameron, J., Veal, E.A., and Morgan, B.A. (2005). Oxidation of a eukaryotic 2-Cys peroxiredoxin is a molecular switch controlling the transcriptional response to increasing levels of hydrogen peroxide. *The Journal of biological chemistry* 280, 23319-23327.

Brewster, J.L., de Valoir, T., Dwyer, N.D., Winter, E., and Gustin, M.C. (1993). An osmosensing signal transduction pathway in yeast. *Science* 259, 1760-1763.

Buck, V., Quinn, J., Soto Pino, T., Martin, H., Saldanha, J., Makino, K., Morgan, B.A., and Millar, J.B. (2001). Peroxide sensors for the fission yeast stress-activated mitogen-activated protein kinase pathway. *Molecular biology of the cell* 12, 407-419.

Budovskaya, Y.V., Stephan, J.S., Reggiori, F., Klionsky, D.J., and Herman, P.K. (2004). The Ras/cAMP-dependent protein kinase signaling pathway regulates an early step of the autophagy process in *Saccharomyces cerevisiae*. *The Journal of biological chemistry* 279, 20663-20671.

Burhans, W.C., and Heintz, N.H. (2009). The cell cycle is a redox cycle: linking phase-specific targets to cell fate. *Free radical biology & medicine* 47, 1282-1293.

Butterfield, D.A., and Boyd-Kimball, D. (2004). Amyloid beta-peptide(1-42) contributes to the oxidative stress and neurodegeneration found in Alzheimer disease brain. *Brain Pathol* 14, 426-432.

Buzek, J., Latonen, L., Kurki, S., Peltonen, K., and Laiho, M. (2002). Redox state of tumor suppressor p53 regulates its sequence-specific DNA binding in DNA-damaged cells by cysteine 277. *Nucleic acids research* 30, 2340-2348.

Capili, A.D., and Lima, C.D. (2007). Taking it step by step: mechanistic insights from structural studies of ubiquitin/ubiquitin-like protein modification pathways. *Current opinion in structural biology* 17, 726-735.

Cash, T.P., Pan, Y., and Simon, M.C. (2007). Reactive oxygen species and cellular oxygen sensing. *Free radical biology & medicine* 43, 1219-1225.

Castillo, E.A., Ayte, J., Chiva, C., Moldon, A., Carrascal, M., Abian, J., Jones, N., and Hidalgo, E. (2002). Diethylmaleate activates the transcription factor Pap1 by covalent modification of critical cysteine residues. *Molecular microbiology* 45, 243-254.

Cerutti, P.A. (1985). Prooxidant states and tumor promotion. *Science* 227, 375-381.

Chae, H.Z., Kim, H.J., Kang, S.W., and Rhee, S.G. (1999). Characterization of three isoforms of mammalian peroxiredoxin that reduce peroxides in the presence of thioredoxin. *Diabetes research and clinical practice* 45, 101-112.

Chakravarthi, S., Jessop, C.E., and Bulleid, N.J. (2006). The role of glutathione in disulphide bond formation and endoplasmic-reticulum-generated oxidative stress. *EMBO reports* 7, 271-275.

Chang, T.S., Jeong, W., Choi, S.Y., Yu, S., Kang, S.W., and Rhee, S.G. (2002). Regulation of peroxiredoxin I activity by Cdc2-mediated phosphorylation. *The Journal of biological chemistry* 277, 25370-25376.

Chen, D., Wilkinson, C.R., Watt, S., Penkett, C.J., Toone, W.M., Jones, N., and Bahler, J. (2008a). Multiple pathways differentially regulate global oxidative stress responses in fission yeast. *Molecular biology of the cell* 19, 308-317.

Chen, Y. (2007). The enzymes in ubiquitin-like post-translational modifications. *Bioscience trends* 1, 16-25.

Chen, Y., McMillan-Ward, E., Kong, J., Israels, S.J., and Gibson, S.B. (2007). Mitochondrial electron-transport-chain inhibitors of complexes I and II induce autophagic cell death mediated by reactive oxygen species. *Journal of cell science* 120, 4155-4166.

Chen, Y., McMillan-Ward, E., Kong, J., Israels, S.J., and Gibson, S.B. (2008b). Oxidative stress induces autophagic cell death independent of apoptosis in transformed and cancer cells. *Cell death and differentiation* 15, 171-182.

Chen, Z.J., and Sun, L.J. (2009). Nonproteolytic functions of ubiquitin in cell signaling. *Molecular cell* 33, 275-286.

Chevtzoff, C., Yoboue, E.D., Galinier, A., Casteilla, L., Daignan-Fornier, B., Rigoulet, M., and Devin, A. (2010). Reactive oxygen species-mediated regulation of mitochondrial biogenesis in the yeast *Saccharomyces cerevisiae*. *The Journal of biological chemistry* 285, 1733-1742.

Chiu, Y.H., Sun, Q., and Chen, Z.J. (2007). E1-L2 activates both ubiquitin and FAT10. *Molecular cell* 27, 1014-1023.

Choi, H., Kim, S., Mukhopadhyay, P., Cho, S., Woo, J., Storz, G., and Ryu, S.E. (2001). Structural basis of the redox switch in the OxyR transcription factor. *Cell* 105, 103-113.

Choi, M.H., Lee, I.K., Kim, G.W., Kim, B.U., Han, Y.H., Yu, D.Y., Park, H.S., Kim, K.Y., Lee, J.S., Choi, C., *et al.* (2005). Regulation of PDGF signalling and vascular remodelling by peroxiredoxin II. *Nature* 435, 347-353.

Cosgrove, J.P., Borish, E.T., Church, D.F., and Pryor, W.A. (1985). The metal-mediated formation of hydroxyl radical by aqueous extracts of cigarette tar. *Biochemical and biophysical research communications* 132, 390-396.

Cuervo, A.M. (2004). Autophagy: in sickness and in health. *Trends in cell biology* 14, 70-77.

D'Autreaux, B., and Toledano, M.B. (2007). ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nature reviews Molecular cell biology* 8, 813-824.

Dasso, M. (2008). Emerging roles of the SUMO pathway in mitosis. *Cell division* 3, 5.

David, Y., Ziv, T., Admon, A., and Navon, A. (2010). The E2 ubiquitin-conjugating enzymes direct polyubiquitination to preferred lysines. *The Journal of biological chemistry* 285, 8595-8604.

Day, A.M., and Veal, E.A. (2010). Hydrogen peroxide-sensitive cysteines in the Sty1 MAPK regulate the transcriptional response to oxidative stress. *The Journal of biological chemistry* 285, 7505-7516.

Degols, G., Shiozaki, K., and Russell, P. (1996). Activation and regulation of the Spc1 stress-activated protein kinase in *Schizosaccharomyces pombe*. *Molecular and cellular biology* 16, 2870-2877.

Delaunay, A., Isnard, A.D., and Toledano, M.B. (2000). H<sub>2</sub>O<sub>2</sub> sensing through oxidation of the Yap1 transcription factor. *The EMBO journal* 19, 5157-5166.

Delaunay, A., Pflieger, D., Barrault, M.B., Vinh, J., and Toledano, M.B. (2002). A thiol peroxidase is an H<sub>2</sub>O<sub>2</sub> receptor and redox-transducer in gene activation. *Cell* 111, 471-481.

Denu, J.M., and Dixon, J.E. (1998). Protein tyrosine phosphatases: mechanisms of catalysis and regulation. *Current opinion in chemical biology* 2, 633-641.



Desterro, J.M., Rodriguez, M.S., and Hay, R.T. (1998). SUMO-1 modification of IkappaBalpha inhibits NF-kappaB activation. *Molecular cell* 2, 233-239.

Desterro, J.M., Thomson, J., and Hay, R.T. (1997). Ubc9 conjugates SUMO but not ubiquitin. *FEBS letters* 417, 297-300.

Dewez, M., Bauer, F., Dieu, M., Raes, M., Vandenhaute, J., and Hermand, D. (2008). The conserved Wobble uridine tRNA thiolase Ctu1-Ctu2 is required to maintain genome integrity. *Proceedings of the National Academy of Sciences of the United States of America* 105, 5459-5464.

DeYulia, G.J., Jr., Carcamo, J.M., Borquez-Ojeda, O., Shelton, C.C., and Golde, D.W. (2005). Hydrogen peroxide generated extracellularly by receptor-ligand interaction facilitates cell signaling. *Proceedings of the National Academy of Sciences of the United States of America* 102, 5044-5049.

Dieckhoff, P., Bolte, M., Sancak, Y., Braus, G.H., and Irniger, S. (2004). Smt3/SUMO and Ubc9 are required for efficient APC/C-mediated proteolysis in budding yeast. *Molecular microbiology* 51, 1375-1387.

Djavaheri-Mergny, M., Amelotti, M., Mathieu, J., Besancon, F., Bauvy, C., Souquere, S., Pierron, G., and Codogno, P. (2006). NF-kappaB activation represses tumor necrosis factor-alpha-induced autophagy. *The Journal of biological chemistry* 281, 30373-30382.

Duda, D.M., Walden, H., Sfondouris, J., and Schulman, B.A. (2005). Structural analysis of Escherichia coli ThiF. *Journal of molecular biology* 349, 774-786.

Dye, B.T., and Schulman, B.A. (2007). Structural mechanisms underlying posttranslational modification by ubiquitin-like proteins. *Annual review of biophysics and biomolecular structure* 36, 131-150.

Eletr, Z.M., Huang, D.T., Duda, D.M., Schulman, B.A., and Kuhlman, B. (2005). E2 conjugating enzymes must disengage from their E1 enzymes before E3-dependent ubiquitin and ubiquitin-like transfer. *Nature structural & molecular biology* 12, 933-934.

Feldman, R.M., Correll, C.C., Kaplan, K.B., and Deshaies, R.J. (1997). A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. *Cell* 91, 221-230.

Fernandes, L., Rodrigues-Pousada, C., and Struhl, K. (1997). Yap, a novel family of eight bZIP proteins in Saccharomyces cerevisiae with distinct biological functions. *Molecular and cellular biology* 17, 6982-6993.

Filomeni, G., Rotilio, G., and Ciriolo, M.R. (2005). Disulfide relays and phosphorylative cascades: partners in redox-mediated signaling pathways. *Cell death and differentiation* 12, 1555-1563.

Finkel, T., and Holbrook, N.J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature* 408, 239-247.

Fomenko, D.E., Koc, A., Agisheva, N., Jacobsen, M., Kaya, A., Malinouski, M., Rutherford, J.C., Siu, K.L., Jin, D.Y., Winge, D.R., *et al.* (2011). Thiol peroxidases mediate specific genome-wide regulation of gene expression in response to hydrogen peroxide. *Proceedings of the National Academy of Sciences of the United States of America* 108, 2729-2734.

Foreman, J., Demidchik, V., Bothwell, J.H., Mylona, P., Miedema, H., Torres, M.A., Linstead, P., Costa, S., Brownlee, C., Jones, J.D., *et al.* (2003). Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature* 422, 442-446.

Fujino, G., Noguchi, T., Takeda, K., and Ichijo, H. (2006). Thioredoxin and protein kinases in redox signaling. *Seminars in cancer biology* 16, 427-435.

Furukawa, K., Mizushima, N., Noda, T., and Ohsumi, Y. (2000). A protein conjugation system in yeast with homology to biosynthetic enzyme reaction of prokaryotes. *The Journal of biological chemistry* 275, 7462-7465.

Gallogly, M.M., and Mieyal, J.J. (2007). Mechanisms of reversible protein glutathionylation in redox signaling and oxidative stress. *Current opinion in pharmacology* 7, 381-391.

Gareau, J.R., and Lima, C.D. (2010). The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition. *Nature reviews Molecular cell biology* 11, 861-871.

Geeraert, C., Ratier, A., Pfisterer, S.G., Perdiz, D., Cantaloube, I., Rouault, A., Pattingre, S., Proikas-Cezanne, T., Codogno, P., and Pous, C. (2010). Starvation-induced hyperacetylation of tubulin is required for the stimulation of autophagy by nutrient deprivation. *The Journal of biological chemistry* 285, 24184-24194.

Geisler, S., Holmstrom, K.M., Skujat, D., Fiesel, F.C., Rothfuss, O.C., Kahle, P.J., and Springer, W. (2010). PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nature cell biology* 12, 119-131.

Geiss-Friedlander, R., and Melchior, F. (2007). Concepts in sumoylation: a decade on. *Nature reviews Molecular cell biology* 8, 947-956.

Geng, J., and Klionsky, D.J. (2008). The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. 'Protein modifications: beyond the usual suspects' review series. *EMBO reports* 9, 859-864.

Ghezzi, P. (2005). Regulation of protein function by glutathionylation. *Free radical research* 39, 573-580.

Girdwood, D., Xirodimas, D.P., and Gordon, C. (2011). The essential functions of NEDD8 are mediated via distinct surface regions, and not by polyneddylation in *Schizosaccharomyces pombe*. *PLoS one* 6, e20089.

Gius, D., Botero, A., Shah, S., and Curry, H.A. (1999). Intracellular oxidation/reduction status in the regulation of transcription factors NF-kappaB and AP-1. *Toxicology letters* 106, 93-106.

Glickman, M.H., and Ciechanover, A. (2002). The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiological reviews* 82, 373-428.

Glotzer, M., Murray, A.W., and Kirschner, M.W. (1991). Cyclin is degraded by the ubiquitin pathway. *Nature* 349, 132-138.

Goehring, A.S., Rivers, D.M., and Sprague, G.F., Jr. (2003a). Attachment of the ubiquitin-related protein Urm1p to the antioxidant protein Ahp1p. *Eukaryotic cell* 2, 930-936.

Goehring, A.S., Rivers, D.M., and Sprague, G.F., Jr. (2003b). Urm1ylation: a ubiquitin-like pathway that functions during invasive growth and budding in yeast. *Molecular biology of the cell* 14, 4329-4341.

Gong, L., and Yeh, E.T. (1999). Identification of the activating and conjugating enzymes of the NEDD8 conjugation pathway. *The Journal of biological chemistry* 274, 12036-12042.

Groettrup, M., Pelzer, C., Schmidtke, G., and Hofmann, K. (2008). Activating the ubiquitin family: UBA6 challenges the field. *Trends in biochemical sciences* 33, 230-237.

Guardavaccaro, D., and Pagano, M. (2004). Oncogenic aberrations of cullin-dependent ubiquitin ligases. *Oncogene* 23, 2037-2049.

Gupta, S., Campbell, D., Derijard, B., and Davis, R.J. (1995). Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science* 267, 389-393.

Gutierrez, G.J., and Ronai, Z. (2006). Ubiquitin and SUMO systems in the regulation of mitotic checkpoints. *Trends in biochemical sciences* 31, 324-332.

Gutscher, M., Sobotta, M.C., Wabnitz, G.H., Ballikaya, S., Meyer, A.J., Samstag, Y., and Dick, T.P. (2009). Proximity-based protein thiol oxidation by H<sub>2</sub>O<sub>2</sub>-scavenging peroxidases. *The Journal of biological chemistry* 284, 31532-31540.

Haglund, K., Sigismund, S., Polo, S., Szymkiewicz, I., Di Fiore, P.P., and Dikic, I. (2003). Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. *Nature cell biology* 5, 461-466.

Hainaut, P., and Milner, J. (1993). Redox modulation of p53 conformation and sequence-specific DNA binding in vitro. *Cancer research* 53, 4469-4473.

Halliwell, B., and Gutteridge, J.M. (1990). Role of free radicals and catalytic metal ions in human disease: an overview. *Methods in enzymology* 186, 1-85.

Hanada, T., Noda, N.N., Satomi, Y., Ichimura, Y., Fujioka, Y., Takao, T., Inagaki, F., and Ohsumi, Y. (2007). The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy. *The Journal of biological chemistry* 282, 37298-37302.

Haracska, L., Torres-Ramos, C.A., Johnson, R.E., Prakash, S., and Prakash, L. (2004). Opposing effects of ubiquitin conjugation and SUMO modification of PCNA on replicational bypass of DNA lesions in *Saccharomyces cerevisiae*. *Molecular and cellular biology* 24, 4267-4274.

Hardeland, U., Steinacher, R., Jiricny, J., and Schar, P. (2002). Modification of the human thymine-DNA glycosylase by ubiquitin-like proteins facilitates enzymatic turnover. *The EMBO journal* **21**, 1456-1464.

Harman, D. (1956). Aging: a theory based on free radical and radiation chemistry. *Journal of gerontology* **11**, 298-300.

Harreman, M., Taschner, M., Sigurdsson, S., Anindya, R., Reid, J., Somesh, B., Kong, S.E., Banks, C.A., Conaway, R.C., Conaway, J.W., *et al.* (2009). Distinct ubiquitin ligases act sequentially for RNA polymerase II polyubiquitylation. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 20705-20710.

Hay, R.T. (2005). SUMO: a history of modification. *Molecular cell* **18**, 1-12.

Heideker, J., Perry, J.J., and Boddy, M.N. (2009). Genome stability roles of SUMO-targeted ubiquitin ligases. *DNA repair* **8**, 517-524.

Herrmann, J., Lerman, L.O., and Lerman, A. (2007). Ubiquitin and ubiquitin-like proteins in protein regulation. *Circulation research* **100**, 1276-1291.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. *Annual review of biochemistry* **67**, 425-479.

Hidalgo, E., and Demple, B. (1994). An iron-sulfur center essential for transcriptional activation by the redox-sensing SoxR protein. *The EMBO journal* **13**, 138-146.

Hochstrasser, M. (2000). Evolution and function of ubiquitin-like protein-conjugation systems. *Nature cell biology* **2**, E153-157.

Hochstrasser, M. (2009). Origin and function of ubiquitin-like proteins. *Nature* **458**, 422-429.

Hoegel, C., Pfander, B., Moldovan, G.L., Pyrowolakis, G., and Jentsch, S. (2002). RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature* **419**, 135-141.

Huang, B., Lu, J., and Bystrom, A.S. (2008). A genome-wide screen identifies genes required for formation of the wobble nucleoside 5-methoxycarbonylmethyl-2-thiouridine in *Saccharomyces cerevisiae*. *RNA* **14**, 2183-2194.

Huang, D.T., Miller, D.W., Mathew, R., Cassell, R., Holton, J.M., Roussel, M.F., and Schulman, B.A. (2004). A unique E1-E2 interaction required for optimal conjugation of the ubiquitin-like protein NEDD8. *Nature structural & molecular biology* **11**, 927-935.

Huang, D.T., Paydar, A., Zhuang, M., Waddell, M.B., Holton, J.M., and Schulman, B.A. (2005). Structural basis for recruitment of Ubc12 by an E2 binding domain in NEDD8's E1. *Molecular cell* **17**, 341-350.

Huang, J., Canadien, V., Lam, G.Y., Steinberg, B.E., Dinauer, M.C., Magalhaes, M.A., Glogauer, M., Grinstein, S., and Brumell, J.H. (2009). Activation of antibacterial autophagy by NADPH oxidases. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 6226-6231.

Huang, L., Kinnucan, E., Wang, G., Beaudenon, S., Howley, P.M., Huibregtse, J.M., and Pavletich, N.P. (1999). Structure of an E6AP-UbcH7 complex: insights into ubiquitination by the E2-E3 enzyme cascade. *Science* **286**, 1321-1326.

Huang, T.T., Nijman, S.M., Mirchandani, K.D., Galardy, P.J., Cohn, M.A., Haas, W., Gygi, S.P., Ploegh, H.L., Bernards, R., and D'Andrea, A.D. (2006). Regulation of monoubiquitinated PCNA by DUB autocleavage. *Nature cell biology* **8**, 339-347.

Humbard, M.A., Miranda, H.V., Lim, J.M., Krause, D.J., Pritz, J.R., Zhou, G., Chen, S., Wells, L., and Maupin-Furlow, J.A. (2010). Ubiquitin-like small archaeal modifier proteins (SAMPs) in *Haloferax volcanii*. *Nature* **463**, 54-60.

Humphries, K.M., Juliano, C., and Taylor, S.S. (2002). Regulation of cAMP-dependent protein kinase activity by glutathionylation. *The Journal of biological chemistry* **277**, 43505-43511.

Hutchins, M.U., Veenhuis, M., and Klionsky, D.J. (1999). Peroxisome degradation in *Saccharomyces cerevisiae* is dependent on machinery of macroautophagy and the Cvt pathway. *Journal of cell science* **112** ( Pt 22), 4079-4087.

Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., and Gotoh, Y. (1997). Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* **275**, 90-94.

Ichimura, Y., Kirisako, T., Takao, T., Satomi, Y., Shimonishi, Y., Ishihara, N., Mizushima, N., Tanida, I., Kominami, E., Ohsumi, M., *et al.* (2000). A ubiquitin-like system mediates protein lipidation. *Nature* 408, 488-492.

Ikeda, F., and Dikic, I. (2008). Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series. *EMBO reports* 9, 536-542.

Ikner, A., and Shiozaki, K. (2005). Yeast signaling pathways in the oxidative stress response. *Mutation research* 569, 13-27.

Imoto, K., Kukidome, D., Nishikawa, T., Matsuhisa, T., Sonoda, K., Fujisawa, K., Yano, M., Motoshima, H., Taguchi, T., Tsuruzoe, K., *et al.* (2006). Impact of mitochondrial reactive oxygen species and apoptosis signal-regulating kinase 1 on insulin signaling. *Diabetes* 55, 1197-1204.

Iwai, K., Naganuma, A., and Kuge, S. (2010). Peroxiredoxin Ahp1 acts as a receptor for alkylhydroperoxides to induce disulfide bond formation in the Cad1 transcription factor. *The Journal of biological chemistry* 285, 10597-10604.

Iyer, L.M., Burroughs, A.M., and Aravind, L. (2006). The prokaryotic antecedents of the ubiquitin-signaling system and the early evolution of ubiquitin-like beta-grasp domains. *Genome biology* 7, R60.

Jahngen-Hodge, J., Obin, M.S., Gong, X., Shang, F., Nowell, T.R., Jr., Gong, J., Abasi, H., Blumberg, J., and Taylor, A. (1997). Regulation of ubiquitin-conjugating enzymes by glutathione following oxidative stress. *The Journal of biological chemistry* 272, 28218-28226.

Jang, H.H., Lee, K.O., Chi, Y.H., Jung, B.G., Park, S.K., Park, J.H., Lee, J.R., Lee, S.S., Moon, J.C., Yun, J.W., *et al.* (2004). Two enzymes in one; two yeast peroxiredoxins display oxidative stress-dependent switching from a peroxidase to a molecular chaperone function. *Cell* 117, 625-635.

Johnson, E.S., and Blobel, G. (1997). Ubc9p is the conjugating enzyme for the ubiquitin-like protein Smt3p. *The Journal of biological chemistry* 272, 26799-26802.

Johnson, E.S., Schwienhorst, I., Dohmen, R.J., and Blobel, G. (1997). The ubiquitin-like protein Smt3p is activated for conjugation to other proteins by an Aos1p/Uba2p heterodimer. *The EMBO journal* 16, 5509-5519.

Kadowaki, H., Nishitoh, H., Urano, F., Sadamitsu, C., Matsuzawa, A., Takeda, K., Masutani, H., Yodoi, J., Urano, Y., Nagano, T., *et al.* (2005). Amyloid beta induces neuronal cell death through ROS-mediated ASK1 activation. *Cell death and differentiation* 12, 19-24.

Kamada, Y., Funakoshi, T., Shintani, T., Nagano, K., Ohsumi, M., and Ohsumi, Y. (2000). Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *The Journal of cell biology* 150, 1507-1513.

Kamata, H., Honda, S., Maeda, S., Chang, L., Hirata, H., and Karin, M. (2005). Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell* 120, 649-661.

Kanki, T., and Klionsky, D.J. (2008). Mitophagy in yeast occurs through a selective mechanism. *The Journal of biological chemistry* 283, 32386-32393.

Kato, T., Jr., Okazaki, K., Murakami, H., Stettler, S., Fantes, P.A., and Okayama, H. (1996). Stress signal, mediated by a Hog1-like MAP kinase, controls sexual development in fission yeast. *FEBS letters* 378, 207-212.

Kawakami, T., Chiba, T., Suzuki, T., Iwai, K., Yamanaka, K., Minato, N., Suzuki, H., Shimbara, N., Hidaka, Y., Osaka, F., *et al.* (2001). NEDD8 recruits E2-ubiquitin to SCF E3 ligase. *The EMBO journal* 20, 4003-4012.

Kennedy, N.J., and Davis, R.J. (2003). Role of JNK in tumor development. *Cell Cycle* 2, 199-201.

Kerscher, O., Felberbaum, R., and Hochstrasser, M. (2006). Modification of proteins by ubiquitin and ubiquitin-like proteins. *Annual review of cell and developmental biology* 22, 159-180.

Kiel, J.A. (2010). Autophagy in unicellular eukaryotes. *Philosophical transactions of the Royal Society of London Series B, Biological sciences* 365, 819-830.

Kim, S.O., Merchant, K., Nudelman, R., Beyer, W.F., Jr., Keng, T., DeAngelo, J., Hausladen, A., and Stamler, J.S. (2002). OxyR: a molecular code for redox-related signaling. *Cell* 109, 383-396.

King, R.W., Deshaies, R.J., Peters, J.M., and Kirschner, M.W. (1996). How proteolysis drives the cell cycle. *Science* 274, 1652-1659.

Kirisako, T., Baba, M., Ishihara, N., Miyazawa, K., Ohsumi, M., Yoshimori, T., Noda, T., and Ohsumi, Y. (1999). Formation process of autophagosome is traced with Apg8/Aut7p in yeast. *The Journal of cell biology* 147, 435-446.

Kirisako, T., Ichimura, Y., Okada, H., Kabeya, Y., Mizushima, N., Yoshimori, T., Ohsumi, M., Takao, T., Noda, T., and Ohsumi, Y. (2000). The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. *The Journal of cell biology* 151, 263-276.

Kissova, I., Deffieu, M., Manon, S., and Camougrand, N. (2004). Uth1p is involved in the autophagic degradation of mitochondria. *The Journal of biological chemistry* 279, 39068-39074.

Kitamura, K., Taki, M., Tanaka, N., and Yamashita, I. (2011). Fission yeast Ubr1 ubiquitin ligase influences the oxidative stress response via degradation of active Pap1 bZIP transcription factor in the nucleus. *Molecular microbiology* 80, 739-755.

Kleiger, G., Saha, A., Lewis, S., Kuhlman, B., and Deshaies, R.J. (2009). Rapid E2-E3 assembly and disassembly enable processive ubiquitylation of cullin-RING ubiquitin ligase substrates. *Cell* 139, 957-968.

Klionsky, D.J. (2005). The molecular machinery of autophagy: unanswered questions. *Journal of cell science* 118, 7-18.

Klionsky, D.J., Cregg, J.M., Dunn, W.A., Jr., Emr, S.D., Sakai, Y., Sandoval, I.V., Sibirny, A., Subramani, S., Thumm, M., Veenhuis, M., *et al.* (2003). A unified nomenclature for yeast autophagy-related genes. *Developmental cell* 5, 539-545.

Komatsu, M., Tanida, I., Ueno, T., Ohsumi, M., Ohsumi, Y., and Kominami, E. (2001). The C-terminal region of an Apg7p/Cvt2p is required for homodimerization and is essential for its E1 activity and E1-E2 complex formation. *The Journal of biological chemistry* 276, 9846-9854.

Kornitzer, D., and Ciechanover, A. (2000). Modes of regulation of ubiquitin-mediated protein degradation. *Journal of cellular physiology* 182, 1-11.

Krepinsky, K., and Leimkuhler, S. (2007). Site-directed mutagenesis of the active site loop of the rhodanese-like domain of the human molybdopterin synthase sulfurase MOCS3. Major differences in substrate specificity between eukaryotic and bacterial homologs. *The FEBS journal* 274, 2778-2787.

Kruse, J.P., and Gu, W. (2009). MSL2 promotes Mdm2-independent cytoplasmic localization of p53. *The Journal of biological chemistry* 284, 3250-3263.

Kudo, N., Taoka, H., Toda, T., Yoshida, M., and Horinouchi, S. (1999). A novel nuclear export signal sensitive to oxidative stress in the fission yeast transcription factor Pap1. *The Journal of biological chemistry* 274, 15151-15158.

Kuge, S., Jones, N., and Nomoto, A. (1997). Regulation of  $\gamma$ AP-1 nuclear localization in response to oxidative stress. *The EMBO journal* 16, 1710-1720.

Kumar, A., Wu, H., Collier-Hyams, L.S., Hansen, J.M., Li, T., Yamoah, K., Pan, Z.Q., Jones, D.P., and Neish, A.S. (2007). Commensal bacteria modulate cullin-dependent signaling via generation of reactive oxygen species. *The EMBO journal* 26, 4457-4466.

Kumar, A., Wu, H., Collier-Hyams, L.S., Kwon, Y.M., Hanson, J.M., and Neish, A.S. (2009). The bacterial fermentation product butyrate influences epithelial signaling via reactive oxygen species-mediated changes in cullin-1 neddylation. *J Immunol* 182, 538-546.

Kurz, T., Chou, Y.C., Willems, A.R., Meyer-Schaller, N., Hecht, M.L., Tyers, M., Peter, M., and Sicheri, F. (2008). Dcn1 functions as a scaffold-type E3 ligase for cullin neddylation. *Molecular cell* 29, 23-35.

Lake, M.W., Wuebbens, M.M., Rajagopalan, K.V., and Schindelin, H. (2001). Mechanism of ubiquitin activation revealed by the structure of a bacterial MoeB-MoaD complex. *Nature* 414, 325-329.

Lambeth, J.D. (2004). NOX enzymes and the biology of reactive oxygen. *Nature reviews Immunology* 4, 181-189.

Landles, C., and Bates, G.P. (2004). Huntingtin and the molecular pathogenesis of Huntington's disease. Fourth in molecular medicine review series. *EMBO reports* 5, 958-963.

Lawrence, C.L., Jones, N., and Wilkinson, C.R. (2009). Stress-induced phosphorylation of *S. pombe* Atf1 abrogates its interaction with F box protein Fbh1. *Current biology : CB* 19, 1907-1911.

Leck, Y.C., Choo, Y.Y., Tan, C.Y., Smith, P.G., and Hagen, T. (2010). Biochemical and cellular effects of inhibiting Nedd8 conjugation. *Biochemical and biophysical research communications* 398, 588-593.

Lee, G.W., Melchior, F., Matunis, M.J., Mahajan, R., Tian, Q., and Anderson, P. (1998a). Modification of Ran GTPase-activating protein by the small ubiquitin-related modifier SUMO-1 requires Ubc9, an E2-type ubiquitin-conjugating enzyme homologue. *The Journal of biological chemistry* 273, 6503-6507.

Lee, I., and Schindelin, H. (2008). Structural insights into E1-catalyzed ubiquitin activation and transfer to conjugating enzymes. *Cell* 134, 268-278.

Lee, I.H., Cao, L., Mostoslavsky, R., Lombard, D.B., Liu, J., Bruns, N.E., Tsokos, M., Alt, F.W., and Finkel, T. (2008). A role for the NAD-dependent deacetylase Sirt1 in the regulation of autophagy. *Proceedings of the National Academy of Sciences of the United States of America* 105, 3374-3379.

Lee, I.H., and Finkel, T. (2009). Regulation of autophagy by the p300 acetyltransferase. *The Journal of biological chemistry* 284, 6322-6328.

Lee, S.R., Kwon, K.S., Kim, S.R., and Rhee, S.G. (1998b). Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. *The Journal of biological chemistry* 273, 15366-15372.

Lee, S.R., Yang, K.S., Kwon, J., Lee, C., Jeong, W., and Rhee, S.G. (2002). Reversible inactivation of the tumor suppressor PTEN by H<sub>2</sub>O<sub>2</sub>. *The Journal of biological chemistry* 277, 20336-20342.

Lehmann, C., Begley, T.P., and Ealick, S.E. (2006). Structure of the *Escherichia coli* ThiS-ThiF complex, a key component of the sulfur transfer system in thiamin biosynthesis. *Biochemistry* 45, 11-19.

Leidecker, O., Matic, I., Mahata, B., Pion, E., and Xirodimas, D.P. (2012). The ubiquitin E1 enzyme Ube1 mediates NEDD8 activation under diverse stress conditions. *Cell Cycle* 11, 1142-1150.

Leidel, S., Pedrioli, P.G., Bucher, T., Brost, R., Costanzo, M., Schmidt, A., Aebersold, R., Boone, C., Hofmann, K., and Peter, M. (2009). Ubiquitin-related modifier Urm1 acts as a sulphur carrier in thiolation of eukaryotic transfer RNA. *Nature* 458, 228-232.

Levine, B., and Kroemer, G. (2008). Autophagy in the pathogenesis of disease. *Cell* 132, 27-42.

Levonen, A.L., Patel, R.P., Brookes, P., Go, Y.M., Jo, H., Parthasarathy, S., Anderson, P.G., and Darley-Usmar, V.M. (2001). Mechanisms of cell signaling by nitric oxide and peroxynitrite: from mitochondria to MAP kinases. *Antioxidants & redox signaling* 3, 215-229.

Li, J., Stouffs, M., Serrander, L., Banfi, B., Bettiol, E., Charnay, Y., Steger, K., Krause, K.H., and Jaconi, M.E. (2006). The NADPH oxidase NOX4 drives cardiac differentiation: Role in regulating cardiac transcription factors and MAP kinase activation. *Molecular biology of the cell* 17, 3978-3988.

Li, M., Brooks, C.L., Wu-Baer, F., Chen, D., Baer, R., and Gu, W. (2003a). Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. *Science* 302, 1972-1975.

Li, Y.P., Chen, Y., Li, A.S., and Reid, M.B. (2003b). Hydrogen peroxide stimulates ubiquitin-conjugating activity and expression of genes for specific E2 and E3 proteins in skeletal muscle myotubes. *American journal of physiology Cell physiology* 285, C806-812.

Liakopoulos, D., Doenges, G., Matuschewski, K., and Jentsch, S. (1998). A novel protein modification pathway related to the ubiquitin system. *The EMBO journal* 17, 2208-2214.

Liochev, S.I., and Fridovich, I. (1992). Fumarase C, the stable fumarase of *Escherichia coli*, is controlled by the soxRS regulon. *Proceedings of the National Academy of Sciences of the United States of America* 89, 5892-5896.

Liu, B., Chen, Y., and St Clair, D.K. (2008). ROS and p53: a versatile partnership. *Free radical biology & medicine* 44, 1529-1535.

Liu, Y., and Min, W. (2002). Thioredoxin promotes ASK1 ubiquitination and degradation to inhibit ASK1-mediated apoptosis in a redox activity-independent manner. *Circulation research* 90, 1259-1266.

Livingstone, C., Patel, G., and Jones, N. (1995). ATF-2 contains a phosphorylation-dependent transcriptional activation domain. *The EMBO journal* 14, 1785-1797.

Loewith, R., Hubberstey, A., and Young, D. (2000). Skh1, the MEK component of the mkh1 signaling pathway in *Schizosaccharomyces pombe*. *Journal of cell science* 113 ( Pt 1), 153-160.

Lois, L.M., and Lima, C.D. (2005). Structures of the SUMO E1 provide mechanistic insights into SUMO activation and E2 recruitment to E1. *The EMBO journal* 24, 439-451.

Lopez-Aviles, S., Grande, M., Gonzalez, M., Helgesen, A.L., Alemany, V., Sanchez-Piris, M., Bachs, O., Millar, J.B., and Aligue, R. (2005). Inactivation of the Cdc25 phosphatase by the stress-activated Srk1 kinase in fission yeast. *Molecular cell* 17, 49-59.

Lopez-Aviles, S., Lambea, E., Moldon, A., Grande, M., Fajardo, A., Rodriguez-Gabriel, M.A., Hidalgo, E., and Aligue, R. (2008). Activation of Srk1 by the mitogen-activated protein kinase Sty1/Spc1 precedes its dissociation from the kinase and signals its degradation. *Molecular biology of the cell* 19, 1670-1679.

Lyapina, S., Cope, G., Shevchenko, A., Serino, G., Tsuge, T., Zhou, C., Wolf, D.A., Wei, N., and Deshaies, R.J. (2001). Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome. *Science* 292, 1382-1385.

Madrid, M., Nunez, A., Soto, T., Vicente-Soler, J., Gacto, M., and Cansado, J. (2007). Stress-activated protein kinase-mediated down-regulation of the cell integrity pathway mitogen-activated protein kinase Pmk1p by protein phosphatases. *Molecular biology of the cell* 18, 4405-4419.

Madrid, M., Soto, T., Franco, A., Paredes, V., Vicente, J., Hidalgo, E., Gacto, M., and Cansado, J. (2004). A cooperative role for Atf1 and Pap1 in the detoxification of the oxidative stress induced by glucose deprivation in *Schizosaccharomyces pombe*. *The Journal of biological chemistry* 279, 41594-41602.

Madrid, M., Soto, T., Khong, H.K., Franco, A., Vicente, J., Perez, P., Gacto, M., and Cansado, J. (2006). Stress-induced response, localization, and regulation of the Pmk1 cell integrity pathway in *Schizosaccharomyces pombe*. *The Journal of biological chemistry* 281, 2033-2043.

Mahajan, R., Delphin, C., Guan, T., Gerace, L., and Melchior, F. (1997). A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell* 88, 97-107.

Marshall, C.J. (1994). MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Current opinion in genetics & development* 4, 82-89.

Matsuzawa, A., and Ichijo, H. (2008). Redox control of cell fate by MAP kinase: physiological roles of ASK1-MAP kinase pathway in stress signaling. *Biochimica et biophysica acta* 1780, 1325-1336.

Matunis, M.J., Coutavas, E., and Blobel, G. (1996). A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *The Journal of cell biology* 135, 1457-1470.

Mendenhall, M.D. (1993). An inhibitor of p34CDC28 protein kinase activity from *Saccharomyces cerevisiae*. *Science* 259, 216-219.

Michael, W.M., and Newport, J. (1998). Coupling of mitosis to the completion of S phase through Cdc34-mediated degradation of Wee1. *Science* 282, 1886-1889.

Millar, J.B., Buck, V., and Wilkinson, M.G. (1995). Pyp1 and Pyp2 PTPases dephosphorylate an osmosensing MAP kinase controlling cell size at division in fission yeast. *Genes & development* 9, 2117-2130.

Miranda, H.V., Nembhard, N., Su, D., Hepowit, N., Krause, D.J., Pritz, J.R., Phillips, C., Soll, D., and Maupin-Furlow, J.A. (2011). E1- and ubiquitin-like proteins provide a direct link between protein conjugation and sulfur transfer in archaea. *Proceedings of the National Academy of Sciences of the United States of America* 108, 4417-4422.

Mizushima, N., Levine, B., Cuervo, A.M., and Klionsky, D.J. (2008). Autophagy fights disease through cellular self-digestion. *Nature* 451, 1069-1075.

Mizushima, N., Noda, T., and Ohsumi, Y. (1999). Apg16p is required for the function of the Apg12p-Apg5p conjugate in the yeast autophagy pathway. *The EMBO journal* 18, 3888-3896.

Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M.D., Klionsky, D.J., Ohsumi, M., and Ohsumi, Y. (1998). A protein conjugation system essential for autophagy. *Nature* 395, 395-398.

Mizushima, N., Yoshimori, T., and Ohsumi, Y. (2002). Mouse Apg10 as an Apg12-conjugating enzyme: analysis by the conjugation-mediated yeast two-hybrid method. *FEBS letters* 532, 450-454.

Mohideen, F., Capili, A.D., Bilimoria, P.M., Yamada, T., Bonni, A., and Lima, C.D. (2009). A molecular basis for phosphorylation-dependent SUMO conjugation by the E2 UBC9. *Nature structural & molecular biology* 16, 945-952.

Mukaiyama, H., Kajiwar, S., Hosomi, A., Giga-Hama, Y., Tanaka, N., Nakamura, T., and Takegawa, K. (2009). Autophagy-deficient *Schizosaccharomyces pombe* mutants undergo partial sporulation during nitrogen starvation. *Microbiology* 155, 3816-3826.

Mukherjee, S., and Orth, K. (2008). Microbiology. A protein pupylation paradigm. *Science* 322, 1062-1063.

Nadeau, P.J., Charette, S.J., Toledano, M.B., and Landry, J. (2007). Disulfide Bond-mediated multimerization of Ask1 and its reduction by thioredoxin-1 regulate H<sub>2</sub>O<sub>2</sub>-induced c-Jun NH<sub>2</sub>-terminal kinase activation and apoptosis. *Molecular biology of the cell* 18, 3903-3913.

Nakai, Y., Nakai, M., and Hayashi, H. (2008). Thio-modification of yeast cytosolic tRNA requires a ubiquitin-related system that resembles bacterial sulfur transfer systems. *The Journal of biological chemistry* 283, 27469-27476.

Nakatogawa, H., Suzuki, K., Kamada, Y., and Ohsumi, Y. (2009). Dynamics and diversity in autophagy mechanisms: lessons from yeast. *Nature reviews Molecular cell biology* 10, 458-467.

Nakayama, K.I., and Nakayama, K. (2006). Ubiquitin ligases: cell-cycle control and cancer. *Nature reviews Cancer* 6, 369-381.

Narendra, D., Tanaka, A., Suen, D.F., and Youle, R.J. (2008). Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *The Journal of cell biology* 183, 795-803.

Nefsky, B., and Beach, D. (1996). Pub1 acts as an E6-AP-like protein ubiquitin ligase in the degradation of cdc25. *The EMBO journal* 15, 1301-1312.

Nemoto, T., Tanida, I., Tanida-Miyake, E., Minematsu-Ikeguchi, N., Yokota, M., Ohsumi, M., Ueno, T., and Kominami, E. (2003). The mouse APG10 homologue, an E2-like enzyme for Apg12p conjugation, facilitates MAP-LC3 modification. *The Journal of biological chemistry* 278, 39517-39526.

Nguyen, A.N., and Shiozaki, K. (1999). Heat-shock-induced activation of stress MAP kinase is regulated by threonine- and tyrosine-specific phosphatases. *Genes & development* 13, 1653-1663.

Nishizawa, M., Kawasumi, M., Fujino, M., and Toh-e, A. (1998). Phosphorylation of sic1, a cyclin-dependent kinase (Cdk) inhibitor, by Cdk including Pho85 kinase is required for its prompt degradation. *Molecular biology of the cell* 9, 2393-2405.

Noda, N.N., Ohsumi, Y., and Inagaki, F. (2009). ATG systems from the protein structural point of view. *Chemical reviews* 109, 1587-1598.

Noda, T., and Ohsumi, Y. (1998). Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. *The Journal of biological chemistry* 273, 3963-3966.

Noguchi, T., Takeda, K., Matsuzawa, A., Saegusa, K., Nakano, H., Gohda, J., Inoue, J., and Ichijo, H. (2005). Recruitment of tumor necrosis factor receptor-associated factor family proteins to apoptosis signal-regulating kinase 1 signalosome is essential for oxidative stress-induced cell death. *The Journal of biological chemistry* 280, 37033-37040.

Noma, A., Sakaguchi, Y., and Suzuki, T. (2009). Mechanistic characterization of the sulfur-relay system for eukaryotic 2-thiouridine biogenesis at tRNA wobble positions. *Nucleic acids research* 37, 1335-1352.



Oberley, T.D. (2002). Oxidative damage and cancer. *The American journal of pathology* 160, 403-408.

Obin, M., Shang, F., Gong, X., Handelman, G., Blumberg, J., and Taylor, A. (1998). Redox regulation of ubiquitin-conjugating enzymes: mechanistic insights using the thiol-specific oxidant diamide. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 12, 561-569.

Okamoto, K., Kondo-Okamoto, N., and Ohsumi, Y. (2009). A landmark protein essential for mitophagy: Atg32 recruits the autophagic machinery to mitochondria. *Autophagy* 5, 1203-1205.

Okazaki, S., Naganuma, A., and Kuge, S. (2005). Peroxiredoxin-mediated redox regulation of the nuclear localization of Yap1, a transcription factor in budding yeast. *Antioxidants & redox signaling* 7, 327-334.

Onodera, J., and Ohsumi, Y. (2004). Ald6p is a preferred target for autophagy in yeast, *Saccharomyces cerevisiae*. *The Journal of biological chemistry* 279, 16071-16076.

Onodera, J., and Ohsumi, Y. (2005). Autophagy is required for maintenance of amino acid levels and protein synthesis under nitrogen starvation. *The Journal of biological chemistry* 280, 31582-31586.

Osaka, F., Kawasaki, H., Aida, N., Saeki, M., Chiba, T., Kawashima, S., Tanaka, K., and Kato, S. (1998). A new NEDD8-ligating system for cullin-4A. *Genes & development* 12, 2263-2268.

Osaka, F., Saeki, M., Katayama, S., Aida, N., Toh, E.A., Kominami, K., Toda, T., Suzuki, T., Chiba, T., Tanaka, K., *et al.* (2000). Covalent modifier NEDD8 is essential for SCF ubiquitin-ligase in fission yeast. *The EMBO journal* 19, 3475-3484.

Pan, Z.Q., Kentsis, A., Dias, D.C., Yamoah, K., and Wu, K. (2004). Nedd8 on cullin: building an expressway to protein destruction. *Oncogene* 23, 1985-1997.

Pankiv, S., Clausen, T.H., Lamark, T., Brech, A., Bruun, J.A., Outzen, H., Overvatn, A., Bjorkoy, G., and Johansen, T. (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *The Journal of biological chemistry* 282, 24131-24145.

Park, H.S., Lee, S.H., Park, D., Lee, J.S., Ryu, S.H., Lee, W.J., Rhee, S.G., and Bae, Y.S. (2004). Sequential activation of phosphatidylinositol 3-kinase, beta Pix, Rac1, and Nox1 in growth factor-induced production of H<sub>2</sub>O<sub>2</sub>. *Molecular and cellular biology* 24, 4384-4394.

Pascual, M.B., Mata-Cabana, A., Florencio, F.J., Lindahl, M., and Cejudo, F.J. (2010). Overoxidation of 2-Cys peroxiredoxin in prokaryotes: cyanobacterial 2-Cys peroxiredoxins sensitive to oxidative stress. *The Journal of biological chemistry* 285, 34485-34492.

Pattingre, S., Espert, L., Biard-Piechaczyk, M., and Codogno, P. (2008). Regulation of macroautophagy by mTOR and Beclin 1 complexes. *Biochimie* 90, 313-323.

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X.H., Mizushima, N., Packer, M., Schneider, M.D., and Levine, B. (2005). Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* 122, 927-939.

Pearce, M.J., Mintseris, J., Ferreyra, J., Gygi, S.P., and Darwin, K.H. (2008). Ubiquitin-like protein involved in the proteasome pathway of *Mycobacterium tuberculosis*. *Science* 322, 1104-1107.

Pedrioli, P.G., Leidel, S., and Hofmann, K. (2008). Urm1 at the crossroad of modifications. 'Protein Modifications: Beyond the Usual Suspects' Review Series. *EMBO reports* 9, 1196-1202.

Petersen, J., and Hagan, I.M. (2005). Polo kinase links the stress pathway to cell cycle control and tip growth in fission yeast. *Nature* 435, 507-512.

Petroski, M.D., and Deshaies, R.J. (2005). Function and regulation of cullin-RING ubiquitin ligases. *Nature reviews Molecular cell biology* 6, 9-20.

Pichler, A., Knipscheer, P., Oberhofer, E., van Dijk, W.J., Korner, R., Olsen, J.V., Jentsch, S., Melchior, F., and Sixma, T.K. (2005). SUMO modification of the ubiquitin-conjugating enzyme E2-25K. *Nature structural & molecular biology* 12, 264-269.

Plafker, K.S., Nguyen, L., Barneche, M., Mirza, S., Crawford, D., and Plafker, S.M. (2010). The ubiquitin-conjugating enzyme UbcM2 can regulate the stability and activity of the antioxidant transcription factor Nrf2. *The Journal of biological chemistry* 285, 23064-23074.

Pollycove, M., and Feinendegen, L.E. (2003). Radiation-induced versus endogenous DNA damage: possible effect of inducible protective responses in mitigating endogenous damage. *Human & experimental toxicology* 22, 290-306; discussion 307, 315-297, 319-223.

Quinn, J., Findlay, V.J., Dawson, K., Millar, J.B., Jones, N., Morgan, B.A., and Toone, W.M. (2002). Distinct regulatory proteins control the graded transcriptional response to increasing H<sub>2</sub>O<sub>2</sub> levels in fission yeast *Schizosaccharomyces pombe*. *Molecular biology of the cell* 13, 805-816.

Rabut, G., and Peter, M. (2008). Function and regulation of protein neddylation. 'Protein modifications: beyond the usual suspects' review series. *EMBO reports* 9, 969-976.

Radoshevich, L., Murrow, L., Chen, N., Fernandez, E., Roy, S., Fung, C., and Debnath, J. (2010). ATG12 conjugation to ATG3 regulates mitochondrial homeostasis and cell death. *Cell* 142, 590-600.

Rainwater, R., Parks, D., Anderson, M.E., Tegtmeyer, P., and Mann, K. (1995). Role of cysteine residues in regulation of p53 function. *Molecular and cellular biology* 15, 3892-3903.

Reggiori, F., and Klionsky, D.J. (2002). Autophagy in the eukaryotic cell. *Eukaryotic cell* 1, 11-21.

Reverter, D., and Lima, C.D. (2005). Insights into E3 ligase activity revealed by a SUMO-RanGAP1-Ubc9-Nup358 complex. *Nature* 435, 687-692.

Rhee, S.G. (2006). Cell signaling. H<sub>2</sub>O<sub>2</sub>, a necessary evil for cell signaling. *Science* 312, 1882-1883.

Rhee, S.G., Bae, Y.S., Lee, S.R., and Kwon, J. (2000). Hydrogen peroxide: a key messenger that modulates protein phosphorylation through cysteine oxidation. *Science's STKE : signal transduction knowledge environment* 2000, pe1.

Rhee, S.G., Chang, T.S., Bae, Y.S., Lee, S.R., and Kang, S.W. (2003). Cellular regulation by hydrogen peroxide. *Journal of the American Society of Nephrology : JASN* 14, S211-215.

Rhee, S.G., Jeong, W., Chang, T.S., and Woo, H.A. (2007). Sulfiredoxin, the cysteine sulfinic acid reductase specific to 2-Cys peroxiredoxin: its discovery, mechanism of action, and biological significance. *Kidney international Supplement*, S3-8.

Rhee, S.G., Kang, S.W., Jeong, W., Chang, T.S., Yang, K.S., and Woo, H.A. (2005). Intracellular messenger function of hydrogen peroxide and its regulation by peroxiredoxins. *Current opinion in cell biology* 17, 183-189.

Rhee, S.G., Woo, H.A., Kil, I.S., and Bae, S.H. (2012). Peroxiredoxin functions as a peroxidase and a regulator and sensor of local peroxides. *The Journal of biological chemistry* 287, 4403-4410.

Ribet, D., Hamon, M., Gouin, E., Nahori, M.A., Impens, F., Neyret-Kahn, H., Gevaert, K., Vandekerckhove, J., Dejean, A., and Cossart, P. (2010). *Listeria monocytogenes* impairs SUMOylation for efficient infection. *Nature* 464, 1192-1195.

Rodrigo-Brenni, M.C., Foster, S.A., and Morgan, D.O. (2010). Catalysis of lysine 48-specific ubiquitin chain assembly by residues in E2 and ubiquitin. *Molecular cell* 39, 548-559.

Rodriguez-Gabriel, M.A., Burns, G., McDonald, W.H., Martin, V., Yates, J.R., 3rd, Bahler, J., and Russell, P. (2003). RNA-binding protein Csx1 mediates global control of gene expression in response to oxidative stress. *The EMBO journal* 22, 6256-6266.

Rodriguez-Gabriel, M.A., and Russell, P. (2005). Distinct signaling pathways respond to arsenite and reactive oxygen species in *Schizosaccharomyces pombe*. *Eukaryotic cell* 4, 1396-1402.

Rodriguez-Gabriel, M.A., Watt, S., Bahler, J., and Russell, P. (2006). Upf1, an RNA helicase required for nonsense-mediated mRNA decay, modulates the transcriptional response to oxidative stress in fission yeast. *Molecular and cellular biology* 26, 6347-6356.

Rodriguez-Hernandez, A., Cordero, M.D., Salviati, L., Artuch, R., Pineda, M., Briones, P., Gomez Izquierdo, L., Cotan, D., Navas, P., and Sanchez-Alcazar, J.A. (2009). Coenzyme Q deficiency triggers mitochondria degradation by mitophagy. *Autophagy* 5, 19-32.

Rodriguez, M.S., Dargemont, C., and Hay, R.T. (2001). SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting. *The Journal of biological chemistry* 276, 12654-12659.

Rubio-Teixeira, M. (2007). Urmylation controls Nil1p and Gln3p-dependent expression of nitrogen-catabolite repressed genes in *Saccharomyces cerevisiae*. *FEBS letters* 581, 541-550.

Rudolph, M.J., Wuebbens, M.M., Rajagopalan, K.V., and Schindelin, H. (2001). Crystal structure of molybdopterin synthase and its evolutionary relationship to ubiquitin activation. *Nature structural biology* 8, 42-46.

Sablina, A.A., Budanov, A.V., Ilyinskaya, G.V., Agapova, L.S., Kravchenko, J.E., and Chumakov, P.M. (2005). The antioxidant function of the p53 tumor suppressor. *Nature medicine* 11, 1306-1313.

Saha, A., and Deshaies, R.J. (2008). Multimodal activation of the ubiquitin ligase SCF by Nedd8 conjugation. *Molecular cell* 32, 21-31.

Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998). Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *The EMBO journal* 17, 2596-2606.

Salinas, A.E., and Wong, M.G. (1999). Glutathione S-transferases--a review. *Current medicinal chemistry* 6, 279-309.

Salminen, A., and Kaarniranta, K. (2009). SIRT1: regulation of longevity via autophagy. *Cellular signalling* 21, 1356-1360.

Samejima, I., Mackie, S., and Fantes, P.A. (1997). Multiple modes of activation of the stress-responsive MAP kinase pathway in fission yeast. *The EMBO journal* 16, 6162-6170.

Sanchez-Piris, M., Posas, F., Alemany, V., Winge, I., Hidalgo, E., Bachs, O., and Aligue, R. (2002). The serine/threonine kinase Cmk2 is required for oxidative stress response in fission yeast. *The Journal of biological chemistry* 277, 17722-17727.

Sanso, M., Gogol, M., Ayte, J., Seidel, C., and Hidalgo, E. (2008). Transcription factors Pcr1 and Atf1 have distinct roles in stress- and Sty1-dependent gene regulation. *Eukaryotic cell* 7, 826-835.

Savitsky, P.A., and Finkel, T. (2002). Redox regulation of Cdc25C. *The Journal of biological chemistry* 277, 20535-20540.

Scherz-Shouval, R., and Elazar, Z. (2007). ROS, mitochondria and the regulation of autophagy. *Trends in cell biology* 17, 422-427.

Scherz-Shouval, R., Shvets, E., Fass, E., Shorer, H., Gil, L., and Elazar, Z. (2007). Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. *The EMBO journal* 26, 1749-1760.

Schmitz, J., Chowdhury, M.M., Hanzelmann, P., Nimtz, M., Lee, E.Y., Schindelin, H., and Leimkuhler, S. (2008). The sulfurtransferase activity of Uba4 presents a link between ubiquitin-like protein conjugation and activation of sulfur carrier proteins. *Biochemistry* 47, 6479-6489.

Schuller, C., Brewster, J.L., Alexander, M.R., Gustin, M.C., and Ruis, H. (1994). The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the *Saccharomyces cerevisiae* CTT1 gene. *The EMBO journal* 13, 4382-4389.

Schulman, B.A., and Harper, J.W. (2009). Ubiquitin-like protein activation by E1 enzymes: the apex for downstream signalling pathways. *Nature reviews Molecular cell biology* 10, 319-331.

Schwob, E., Bohm, T., Mendenhall, M.D., and Nasmyth, K. (1994). The B-type cyclin kinase inhibitor p40<sup>SIC1</sup> controls the G1 to S transition in *S. cerevisiae*. *Cell* 79, 233-244.

Sengar, A.S., Markley, N.A., Marini, N.J., and Young, D. (1997). Mkh1, a MEK kinase required for cell wall integrity and proper response to osmotic and temperature stress in *Schizosaccharomyces pombe*. *Molecular and cellular biology* 17, 3508-3519.

Shayeghi, M., Doe, C.L., Tavassoli, M., and Watts, F.Z. (1997). Characterisation of *Schizosaccharomyces pombe* rad31, a UBA-related gene required for DNA damage tolerance. *Nucleic acids research* 25, 1162-1169.

Shieh, J.C., Wilkinson, M.G., Buck, V., Morgan, B.A., Makino, K., and Millar, J.B. (1997). The Mcs4 response regulator coordinately controls the stress-activated Wak1-Wis1-Sty1 MAP kinase pathway and fission yeast cell cycle. *Genes & development* 11, 1008-1022.

Shieh, J.C., Wilkinson, M.G., and Millar, J.B. (1998). The Win1 mitotic regulator is a component of the fission yeast stress-activated Sty1 MAPK pathway. *Molecular biology of the cell* 9, 311-322.

Shin, J.A., Choi, E.S., Kim, H.S., Ho, J.C., Watts, F.Z., Park, S.D., and Jang, Y.K. (2005). SUMO modification is involved in the maintenance of heterochromatin stability in fission yeast. *Molecular cell* 19, 817-828.

Shintani, T., and Klionsky, D.J. (2004). Autophagy in health and disease: a double-edged sword. *Science* 306, 990-995.

Shintani, T., Mizushima, N., Ogawa, Y., Matsuura, A., Noda, T., and Ohsumi, Y. (1999). Apg10p, a novel protein-conjugating enzyme essential for autophagy in yeast. *The EMBO journal* 18, 5234-5241.

Shiozaki, K., and Russell, P. (1995). Cell-cycle control linked to extracellular environment by MAP kinase pathway in fission yeast. *Nature* 378, 739-743.

Shiozaki, K., Shiozaki, M., and Russell, P. (1997). Mcs4 mitotic catastrophe suppressor regulates the fission yeast cell cycle through the Wlk1-Wis1-Spc1 kinase cascade. *Molecular biology of the cell* 8, 409-419.

Shiozaki, K., Shiozaki, M., and Russell, P. (1998). Heat stress activates fission yeast Spc1/Styl MAPK by a MEKK-independent mechanism. *Molecular biology of the cell* 9, 1339-1349.

Skowyra, D., Craig, K.L., Tiers, M., Elledge, S.J., and Harper, J.W. (1997). F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell* 91, 209-219.

Smith, D.A., Toone, W.M., Chen, D., Bahler, J., Jones, N., Morgan, B.A., and Quinn, J. (2002). The *Srk1* protein kinase is a target for the *Sty1* stress-activated MAPK in fission yeast. *The Journal of biological chemistry* 277, 33411-33421.

Song, J., Durrin, L.K., Wilkinson, T.A., Krontiris, T.G., and Chen, Y. (2004). Identification of a SUMO-binding motif that recognizes SUMO-modified proteins. *Proceedings of the National Academy of Sciences of the United States of America* 101, 14373-14378.

Song, L., and Rape, M. (2008). Reverse the curse--the role of deubiquitination in cell cycle control. *Current opinion in cell biology* 20, 156-163.

Soucy, T.A., Smith, P.G., and Rolfe, M. (2009). Targeting NEDD8-activated cullin-RING ligases for the treatment of cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research* 15, 3912-3916.

Staniek, K., and Nohl, H. (2000). Are mitochondria a permanent source of reactive oxygen species? *Biochimica et biophysica acta* 1460, 268-275.

Steffan, J.S., Agrawal, N., Pallos, J., Rockabrand, E., Trotman, L.C., Slepko, N., Illes, K., Lukacovich, T., Zhu, Y.Z., Cattaneo, E., *et al.* (2004). SUMO modification of Huntingtin and Huntington's disease pathology. *Science* 304, 100-104.

Stegmeier, F., Rape, M., Draviam, V.M., Nalepa, G., Sowa, M.E., Ang, X.L., McDonald, E.R., 3rd, Li, M.Z., Hannon, G.J., Sorger, P.K., *et al.* (2007). Anaphase initiation is regulated by antagonistic ubiquitination and deubiquitination activities. *Nature* 446, 876-881.

Stephan, J.S., Yeh, Y.Y., Ramachandran, V., Deminoff, S.J., and Herman, P.K. (2009). The Tor and PKA signaling pathways independently target the Atg1/Atg13 protein kinase complex to control autophagy. *Proceedings of the National Academy of Sciences of the United States of America* 106, 17049-17054.

Storz, G., Tartaglia, L.A., and Ames, B.N. (1990). Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. *Science* 248, 189-194.

Sugawara, K., Suzuki, N.N., Fujioka, Y., Mizushima, N., Ohsumi, Y., and Inagaki, F. (2004). The crystal structure of microtubule-associated protein light chain 3, a mammalian homologue of *Saccharomyces cerevisiae* Atg8. *Genes to cells : devoted to molecular & cellular mechanisms* 9, 611-618.

Sugiura, R., Toda, T., Dhut, S., Shuntoh, H., and Kuno, T. (1999). The MAPK kinase *Pek1* acts as a phosphorylation-dependent molecular switch. *Nature* 399, 479-483.

Sun, X.Z., Vinci, C., Makmura, L., Han, S., Tran, D., Nguyen, J., Hamann, M., Grazziani, S., Sheppard, S., Gutova, M., *et al.* (2003). Formation of disulfide bond in p53 correlates with inhibition of DNA binding and tetramerization. *Antioxidants & redox signaling* 5, 655-665.

Sun, Z.W., and Allis, C.D. (2002). Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. *Nature* 418, 104-108.

Sundaresan, M., Yu, Z.X., Ferrans, V.J., Irani, K., and Finkel, T. (1995). Requirement for generation of H<sub>2</sub>O<sub>2</sub> for platelet-derived growth factor signal transduction. *Science* 270, 296-299.

Suzuki, K., Kirisako, T., Kamada, Y., Mizushima, N., Noda, T., and Ohsumi, Y. (2001). The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. *The EMBO journal* 20, 5971-5981.

Suzuki, N.N., Yoshimoto, K., Fujioka, Y., Ohsumi, Y., and Inagaki, F. (2005). The crystal structure of plant ATG12 and its biological implication in autophagy. *Autophagy* 1, 119-126.

Svejstrup, J.Q. (2007). Elongator complex: how many roles does it play? *Current opinion in cell biology* 19, 331-336.

Swords, R.T., Kelly, K.R., Smith, P.G., Garnsey, J.J., Mahalingam, D., Medina, E., Oberheu, K., Padmanabhan, S., O'Dwyer, M., Nawrocki, S.T., *et al.* (2010). Inhibition of NEDD8-activating enzyme: a novel approach for the treatment of acute myeloid leukemia. *Blood* 115, 3796-3800.

Taherbhoy, A.M., Tait, S.W., Kaiser, S.E., Williams, A.H., Deng, A., Nourse, A., Hammel, M., Kurinov, I., Rock, C.O., Green, D.R., *et al.* (2011). Atg8 transfer from Atg7 to Atg3: a distinctive E1-E2 architecture and mechanism in the autophagy pathway. *Molecular cell* 44, 451-461.

Takada, H., Nishimura, M., Asayama, Y., Mannse, Y., Ishiwata, S., Kita, A., Doi, A., Nishida, A., Kai, N., Moriuchi, S., *et al.* (2007). Atf1 is a target of the mitogen-activated protein kinase Pmk1 and regulates cell integrity in fission yeast. *Molecular biology of the cell* 18, 4794-4802.

Takeda, K., and Ichijo, H. (2002). Neuronal p38 MAPK signalling: an emerging regulator of cell fate and function in the nervous system. *Genes to cells : devoted to molecular & cellular mechanisms* 7, 1099-1111.

Takeda, T., Toda, T., Kominami, K., Kohnosu, A., Yanagida, M., and Jones, N. (1995). *Schizosaccharomyces pombe atf1+* encodes a transcription factor required for sexual development and entry into stationary phase. *The EMBO journal* 14, 6193-6208.

Tal, R., Winter, G., Ecker, N., Klionsky, D.J., and Abeliovich, H. (2007). Aup1p, a yeast mitochondrial protein phosphatase homolog, is required for efficient stationary phase mitophagy and cell survival. *The Journal of biological chemistry* 282, 5617-5624.

Tanida, I., Mizushima, N., Kiyooka, M., Ohsumi, M., Ueno, T., Ohsumi, Y., and Kominami, E. (1999). Apg7p/Cvt2p: A novel protein-activating enzyme essential for autophagy. *Molecular biology of the cell* 10, 1367-1379.

Tateishi, K., Omata, M., Tanaka, K., and Chiba, T. (2001). The NEDD8 system is essential for cell cycle progression and morphogenetic pathway in mice. *The Journal of cell biology* 155, 571-579.

Tobiume, K., Matsuzawa, A., Takahashi, T., Nishitoh, H., Morita, K., Takeda, K., Minowa, O., Miyazono, K., Noda, T., and Ichijo, H. (2001). ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. *EMBO reports* 2, 222-228.

Toda, T., Dhut, S., Superti-Furga, G., Gotoh, Y., Nishida, E., Sugiura, R., and Kuno, T. (1996). The fission yeast pmk1+ gene encodes a novel mitogen-activated protein kinase homolog which regulates cell integrity and functions coordinately with the protein kinase C pathway. *Molecular and cellular biology* 16, 6752-6764.

Toda, T., Shimanuki, M., and Yanagida, M. (1991). Fission yeast genes that confer resistance to staurosporine encode an AP-1-like transcription factor and a protein kinase related to the mammalian ERK1/MAP2 and budding yeast FUS3 and KSS1 kinases. *Genes & development* 5, 60-73.

Tokgoz, Z., Siepmann, T.J., Streich, F., Jr., Kumar, B., Klein, J.M., and Haas, A.L. (2012). E1-E2 interactions in ubiquitin and Nedd8 ligation pathways. *The Journal of biological chemistry* 287, 311-321.

Tolbert, B.S., Tajc, S.G., Webb, H., Snyder, J., Nielsen, J.E., Miller, B.L., and Basavappa, R. (2005). The active site cysteine of ubiquitin-conjugating enzymes has a significantly elevated pK<sub>a</sub>: functional implications. *Biochemistry* 44, 16385-16391.

Toone, W.M., Kuge, S., Samuels, M., Morgan, B.A., Toda, T., and Jones, N. (1998). Regulation of the fission yeast transcription factor Pap1 by oxidative stress: requirement for the nuclear

export factor Crm1 (Exportin) and the stress-activated MAP kinase Sty1/Spc1. *Genes & development* 12, 1453-1463.

Tsukada, M., and Ohsumi, Y. (1993). Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae*. *FEBS letters* 333, 169-174.

Turrens, J.F. (1997). Superoxide production by the mitochondrial respiratory chain. *Bioscience reports* 17, 3-8.

Ushio-Fukai, M. (2006). Redox signaling in angiogenesis: role of NADPH oxidase. *Cardiovascular research* 71, 226-235.

Van der Veen, A.G., Schorpp, K., Schlieker, C., Buti, L., Damon, J.R., Spooner, E., Ploegh, H.L., and Jentsch, S. (2011). Role of the ubiquitin-like protein Urm1 as a noncanonical lysine-directed protein modifier. *Proceedings of the National Academy of Sciences of the United States of America* 108, 1763-1770.

van Wijk, S.J., and Timmers, H.T. (2010). The family of ubiquitin-conjugating enzymes (E2s): deciding between life and death of proteins. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 24, 981-993.

Vandenbroucke, K., Robbens, S., Vandepoele, K., Inze, D., Van de Peer, Y., and Van Breusegem, F. (2008). Hydrogen peroxide-induced gene expression across kingdoms: a comparative analysis. *Molecular biology and evolution* 25, 507-516.

Veal, E.A., Day, A.M., and Morgan, B.A. (2007). Hydrogen peroxide sensing and signaling. *Molecular cell* 26, 1-14.

Veal, E.A., Findlay, V.J., Day, A.M., Bozonet, S.M., Evans, J.M., Quinn, J., and Morgan, B.A. (2004). A 2-Cys peroxiredoxin regulates peroxide-induced oxidation and activation of a stress-activated MAP kinase. *Molecular cell* 15, 129-139.

Velu, C.S., Niture, S.K., Doneanu, C.E., Pattabiraman, N., and Srivenugopal, K.S. (2007). Human p53 is inhibited by glutathionylation of cysteines present in the proximal DNA-binding domain during oxidative stress. *Biochemistry* 46, 7765-7780.

Verma, R., Feldman, R.M., and Deshaies, R.J. (1997). SIC1 is ubiquitinated in vitro by a pathway that requires CDC4, CDC34, and cyclin/CDK activities. *Molecular biology of the cell* 8, 1427-1437.

Vivancos, A.P., Castillo, E.A., Biteau, B., Nicot, C., Ayte, J., Toledano, M.B., and Hidalgo, E. (2005). A cysteine-sulfinic acid in peroxiredoxin regulates H<sub>2</sub>O<sub>2</sub>-sensing by the antioxidant Pap1 pathway. *Proceedings of the National Academy of Sciences of the United States of America* 102, 8875-8880.

Vivancos, A.P., Castillo, E.A., Jones, N., Ayte, J., and Hidalgo, E. (2004). Activation of the redox sensor Pap1 by hydrogen peroxide requires modulation of the intracellular oxidant concentration. *Molecular microbiology* 52, 1427-1435.

Vivancos, A.P., Jara, M., Zuin, A., Sanso, M., and Hidalgo, E. (2006). Oxidative stress in *Schizosaccharomyces pombe*: different H<sub>2</sub>O<sub>2</sub> levels, different response pathways. *Molecular genetics and genomics : MGG* 276, 495-502.

Walden, H., Podgorski, M.S., and Schulman, B.A. (2003). Insights into the ubiquitin transfer cascade from the structure of the activating enzyme for NEDD8. *Nature* 422, 330-334.

Wang, F., Liu, M., Qiu, R., and Ji, C. (2011). The dual role of ubiquitin-like protein Urm1 as a protein modifier and sulfur carrier. *Protein & cell* 2, 612-619.

Wang, Y., Gulis, G., Buckner, S., Johnson, P.C., Sullivan, D., Busenlehner, L., and Marcus, S. (2010). The MAP kinase Pmk1 and protein kinase A are required for rotenone resistance in the fission yeast, *Schizosaccharomyces pombe*. *Biochemical and biophysical research communications* 399, 123-128.

Waskiewicz, A.J., and Cooper, J.A. (1995). Mitogen and stress response pathways: MAP kinase cascades and phosphatase regulation in mammals and yeast. *Current opinion in cell biology* 7, 798-805.

Watanabe, Y., and Yamamoto, M. (1996). *Schizosaccharomyces pombe* pcr1<sup>+</sup> encodes a CREB/ATF protein involved in regulation of gene expression for sexual development. *Molecular and cellular biology* 16, 704-711.

Wei, Y., Pattingre, S., Sinha, S., Bassik, M., and Levine, B. (2008). JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. *Molecular cell* 30, 678-688.

Welchman, R.L., Gordon, C., and Mayer, R.J. (2005). Ubiquitin and ubiquitin-like proteins as multifunctional signals. *Nature reviews Molecular cell biology* 6, 599-609.

Wilkinson, M.G., Samuels, M., Takeda, T., Toone, W.M., Shieh, J.C., Toda, T., Millar, J.B., and Jones, N. (1996). The Atf1 transcription factor is a target for the Sty1 stress-activated MAP kinase pathway in fission yeast. *Genes & development* 10, 2289-2301.

Wohlschlegel, J.A., Johnson, E.S., Reed, S.I., and Yates, J.R., 3rd (2004). Global analysis of protein sumoylation in *Saccharomyces cerevisiae*. *The Journal of biological chemistry* 279, 45662-45668.

Woo, H.A., Yim, S.H., Shin, D.H., Kang, D., Yu, D.Y., and Rhee, S.G. (2010). Inactivation of peroxiredoxin I by phosphorylation allows localized H<sub>2</sub>O<sub>2</sub> accumulation for cell signaling. *Cell* 140, 517-528.

Wood, Z.A., Poole, L.B., and Karplus, P.A. (2003a). Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science* 300, 650-653.

Wood, Z.A., Schroder, E., Robin Harris, J., and Poole, L.B. (2003b). Structure, mechanism and regulation of peroxiredoxins. *Trends in biochemical sciences* 28, 32-40.

Wu, J.J., Quijano, C., Chen, E., Liu, H., Cao, L., Fergusson, M.M., Rovira, I., Gutkind, S., Daniels, M.P., Komatsu, M., *et al.* (2009). Mitochondrial dysfunction and oxidative stress mediate the physiological impairment induced by the disruption of autophagy. *Aging* 1, 425-437.

Wu, K., Yamoah, K., Dolios, G., Gan-Erdene, T., Tan, P., Chen, A., Lee, C.G., Wei, N., Wilkinson, K.D., Wang, R., *et al.* (2003a). DEN1 is a dual function protease capable of processing the C terminus of Nedd8 and deconjugating hyper-neddylated CUL1. *The Journal of biological chemistry* 278, 28882-28891.

Wu, P.Y., Hanlon, M., Eddins, M., Tsui, C., Rogers, R.S., Jensen, J.P., Matunis, M.J., Weissman, A.M., Wolberger, C., and Pickart, C.M. (2003b). A conserved catalytic residue in the ubiquitin-conjugating enzyme family. *The EMBO journal* 22, 5241-5250.

Xi, J., Ge, Y., Kinsland, C., McLafferty, F.W., and Begley, T.P. (2001). Biosynthesis of the thiazole moiety of thiamin in *Escherichia coli*: identification of an acyl disulfide-linked protein-protein conjugate that is functionally analogous to the ubiquitin/E1 complex. *Proceedings of the National Academy of Sciences of the United States of America* 98, 8513-8518.

Xie, Z., and Klionsky, D.J. (2007). Autophagosome formation: core machinery and adaptations. *Nature cell biology* 9, 1102-1109.

Xie, Z., Nair, U., and Klionsky, D.J. (2008). Atg8 controls phagophore expansion during autophagosome formation. *Molecular biology of the cell* 19, 3290-3298.

Xirodimas, D.P. (2008). Novel substrates and functions for the ubiquitin-like molecule NEDD8. *Biochemical Society transactions* 36, 802-806.

Xirodimas, D.P., Saville, M.K., Bourdon, J.C., Hay, R.T., and Lane, D.P. (2004). Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. *Cell* 118, 83-97.

Xu, J., Zhang, J., Wang, L., Zhou, J., Huang, H., Wu, J., Zhong, Y., and Shi, Y. (2006). Solution structure of Urm1 and its implications for the origin of protein modifiers. *Proceedings of the National Academy of Sciences of the United States of America* 103, 11625-11630.

Xu, Z., Lam, L.S., Lam, L.H., Chau, S.F., Ng, T.B., and Au, S.W. (2008). Molecular basis of the redox regulation of SUMO proteases: a protective mechanism of intermolecular disulfide linkage against irreversible sulfhydryl oxidation. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 22, 127-137.

Yamada, K., Nakagawa, C.W., and Mutoh, N. (1999). *Schizosaccharomyces pombe* homologue of glutathione peroxidase, which does not contain selenocysteine, is induced by several stresses and works as an antioxidant. *Yeast* 15, 1125-1132.

Yamada, Y., Suzuki, N.N., Hanada, T., Ichimura, Y., Kumeta, H., Fujioka, Y., Ohsumi, Y., and Inagaki, F. (2007). The crystal structure of Atg3, an autophagy-related ubiquitin carrier protein (E2) enzyme that mediates Atg8 lipidation. *The Journal of biological chemistry* 282, 8036-8043.

Yarian, C., Townsend, H., Czystkowski, W., Sochacka, E., Malkiewicz, A.J., Guenther, R., Miskiewicz, A., and Agris, P.F. (2002). Accurate translation of the genetic code depends on tRNA modified nucleosides. *The Journal of biological chemistry* 277, 16391-16395.

Yorimitsu, T., and Klionsky, D.J. (2005). Autophagy: molecular machinery for self-eating. *Cell death and differentiation* 12 *Suppl* 2, 1542-1552.

Yu, L., Wan, F., Dutta, S., Welsh, S., Liu, Z., Freundt, E., Baehrecke, E.H., and Lenardo, M. (2006). Autophagic programmed cell death by selective catalase degradation. *Proceedings of the National Academy of Sciences of the United States of America* 103, 4952-4957.

Zaitsevskaya-Carter, T., and Cooper, J.A. (1997). Spm1, a stress-activated MAP kinase that regulates morphogenesis in *S.pombe*. *The EMBO journal* 16, 1318-1331.

Zhang, H., Bosch-Marce, M., Shimoda, L.A., Tan, Y.S., Baek, J.H., Wesley, J.B., Gonzalez, F.J., and Semenza, G.L. (2008). Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. *The Journal of biological chemistry* 283, 10892-10903.

Zhao, J. (2007). Sumoylation regulates diverse biological processes. *Cellular and molecular life sciences : CMLS* 64, 3017-3033.

Zheng, M., Aslund, F., and Storz, G. (1998). Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* 279, 1718-1721.

Zheng, N., Wang, P., Jeffrey, P.D., and Pavletich, N.P. (2000). Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. *Cell* 102, 533-539.

Zhou, W., Ryan, J.J., and Zhou, H. (2004). Global analyses of sumoylated proteins in *Saccharomyces cerevisiae*. Induction of protein sumoylation by cellular stresses. *The Journal of biological chemistry* 279, 32262-32268.

Zhou, X., Ma, Y., Sugiura, R., Kobayashi, D., Suzuki, M., Deng, L., and Kuno, T. (2010). MAP kinase kinase kinase (MAPKKK)-dependent and -independent activation of Sty1 stress MAPK in fission yeast. *The Journal of biological chemistry* 285, 32818-32823.

Zuin, A., Carmona, M., Morales-Ivorra, I., Gabrielli, N., Vivancos, A.P., Ayte, J., and Hidalgo, E. (2010). Lifespan extension by calorie restriction relies on the Sty1 MAP kinase stress pathway. *The EMBO journal* 29, 981-991.

Zuin, A., Gabrielli, N., Calvo, I.A., Garcia-Santamarina, S., Hoe, K.L., Kim, D.U., Park, H.O., Hayles, J., Ayte, J., and Hidalgo, E. (2008). Mitochondrial dysfunction increases oxidative stress and decreases chronological life span in fission yeast. *PLoS one* 3, e2842.



**Table 1.1. Ubiquitin/Ubl Proteins and Their Conjugating Enzymes**

In addition to ubiquitin, there are many UbIs found in higher eukaryotes. These proteins regulate numerous important processes in the cell. Many UbIs, and their conjugating enzymes, are conserved in lower organisms such as *S. pombe*. Ubiquitin/Ubl conjugation pathways investigated in this thesis are shown in regular font; other UbIs are shown in italics.

Modifier	E1	E2	Functions	Conserved in <i>S. pombe</i> ?
Ubiquitin	Uba1 (and Uba6 in mammals)	Many	Mediates protein degradation. Critical for cell cycle progression. Also mediates receptor endocytosis, DNA repair, and epigenetic regulation of gene expression via chromatin modification.	Yes
SUMO	Uba2	Ubc9	Mainly nuclear. Regulates transcription, DNA replication and repair, nuclear import/export, chromatin structure.	Yes
NEDD8	Uba3 (and Uba1 in mammals)	Ubc12	Modifies CRLs to facilitate ubiquitination. Limited additional substrates (e.g. p53)	Yes
Urm1	Uba4	?	Sulphur transfer to tRNAs. Has roles in responses to oxidative stress and nutrient depletion.	Yes
Atg12	Atg7	Atg10	Autophagosome formation. Conjugated to Atg5. Facilitates Atg8 conjugation to PE.	Yes
Atg8	Atg7	Atg3	Autophagosome formation. Conjugated to PE. Also functions in selective autophagy.	Yes
<i>Hub1</i>	?	?	<i>Pre-mRNA splicing.</i>	<i>Yes</i>
<i>FAT10</i>	<i>Uba6</i>	?	<i>Mediates protein degradation. May regulate cell proliferation and apoptosis.</i>	<i>No</i>
<i>ISP15</i>	<i>Uba7</i>	<i>UbcH8</i>	<i>Regulates immune responses and cytokine production. Expression induced by cytokines.</i>	<i>No</i>
<i>Fub1</i>	?	?	<i>Inhibition of immune responses and inflammation.</i>	<i>No</i>

Table 2.1. <i>S. pombe</i> strains used in this study.		
Strain	Genotype	Source
AD13	<i>h<sup>-</sup> sty1(3Pk):ura4<sup>+</sup> ade6-M210 his7-366 leu1-32</i>	Gift from A. Day
AD22	<i>h<sup>-</sup> sty1::his7<sup>+</sup> ade6-M210 his7-366 leu1-32 ura4-D18</i>	Gift from A. Day
BG_0465	<i>h<sup>-</sup> atg12::KanMX6 ade6-M210 leu1-32 ura4-D18</i>	Gift from S. Whitehall
BG_0880	<i>h<sup>-</sup> atg10::KanMX6 ade6-M210 leu1-32 ura4-D18</i>	Gift from S. Whitehall
BG_2361	<i>h<sup>-</sup> ubc15::KanMX6 ade6-M210 leu1-32 ura4-D18</i>	Gift from S. Whitehall
BG_4808	<i>h<sup>-</sup> urm1::KanMX6 ade6-M210 leu1-32 ura4-D18</i>	Gift from S. Whitehall
CHP428	<i>h<sup>+</sup> ade6-M210 his7-366 leu1-32 ura4-D18</i>	Gift from C. Hoffman
CHP429	<i>h<sup>-</sup> ade6-M210 his7-366 leu1-32 ura4-D18</i>	Gift from C. Hoffman
DH20	<i>h<sup>-</sup> ade6-M210 leu1-32 ura4-D18</i>	Gift from D. Hermand (Dewez <i>et al.</i> , 2008)
DH252	<i>h<sup>-</sup> ctu1::KanMX6 ade6-M210 leu1-32 ura4-D18</i>	Gift from D. Hermand (Dewez <i>et al.</i> , 2008)
DH237	<i>h<sup>-</sup> ctu2::KanMX6 ade6-M210 leu1-32 ura4-D18</i>	Gift from D. Hermand (Dewez <i>et al.</i> , 2008)
JP178	<i>h<sup>-</sup> pap1::ura4<sup>+</sup></i>	Gift from J. Quinn
JP180	<i>h<sup>+</sup> atf1HA6H:ura4<sup>+</sup></i>	Gift from J. Quinn
JT268	<i>h<sup>-</sup> atg8NGFP</i>	Gift from M. Yamamoto (Kohda <i>et al.</i> , 2007)
MF01	<i>h<sup>+</sup> atg7(3Pk):ura4<sup>+</sup> ade6-M210 his7-366 leu1-32 ura4-D18</i>	This study
MF03	<i>h<sup>+</sup> atg4(3Pk):ura4<sup>+</sup> ade6-M210 his7-366 leu1-32 ura4-D18</i>	This study
MF06	<i>h<sup>+</sup> atg5(3Pk):ura4<sup>+</sup> ade6-M210 his7-366 leu1-32 ura4-D18</i>	This study
MF09	<i>h<sup>+</sup> atg3(3Pk):ura4<sup>+</sup> ade6-M210 his7-366 leu1-32 ura4-D18</i>	This study
MF11	<i>h<sup>+</sup> atg10(3Pk):ura4<sup>+</sup> ade6-M210 his7-366 leu1-32 ura4-D18</i>	This study
MF13	<i>h<sup>-</sup> atg12::KanMX6</i>	This study
MF15	<i>h<sup>-</sup> atg10::KanMX6</i>	This study
MF20	<i>h<sup>+</sup> atg4(3Pk):ura4<sup>+</sup></i>	This study
MF23	<i>h<sup>+</sup> uba4(3Pk):ura4<sup>+</sup> ade6-M210 his7-366 leu1-32 ura4-D18</i>	This study
MF25	<i>h<sup>-</sup> urm1::KanMX6 ade6-M210 his7-366 leu1-32 ura4-D18</i>	This study
MF31	<i>h<sup>-</sup> atg10::KanMX6 atg8NGFP</i>	This study
MF43	<i>h<sup>-</sup> atg12::KanMX6 atg8NGFP</i>	This study
MF44	<i>h<sup>-</sup> urm1::KanMX6</i>	This study
MF45	<i>h<sup>+</sup> urm1::KanMX6 sty1(3Pk):ura4<sup>+</sup> ade6-M210</i>	This study
MF46	<i>h<sup>-</sup> atg5(3Pk):ura4<sup>+</sup> atg12::KanMX6</i>	This study

MF47	<i>h<sup>+</sup> xpo2(3Pk):ura4<sup>+</sup> ade6-M210 his7-366 leu1-32 ura4-D18</i>	This study
MF49	<i>h<sup>-</sup> xpo2(3Pk):ura4<sup>+</sup> urm1::KanMX6 ade6-M210 his7-366 leu1-32 ura4-D18</i>	This study
MF51	<i>h<sup>-</sup> pREP2atg10(3Pk):ura4<sup>+</sup> ade6-M210 his7-366 leu1-32 ura4-D18</i>	This study
MF54	<i>h<sup>+</sup> pREP41atg3:LEU2 atg7(3Pk):ura4<sup>+</sup> ade6-M210 his7-366 leu1-32 ura4-D18</i>	This study
MF55	<i>h<sup>-</sup> pREP41atg10<sup>C131S</sup>(3Pk): LEU2 ade6-M210 his7-366 leu1-32 ura4-D18</i>	This study
MF58	<i>h<sup>+</sup> ubc15(3Pk):ura4<sup>+</sup> ade6-M210 his7-366 leu1-32 ura4-D18</i>	This study
MF73	<i>h<sup>+</sup> ubc15::KanMX6 ade6-M210 his7-366 leu1-32 ura4-D18</i>	This study
MF75	<i>h<sup>-</sup> urm1::KanMX6 pap1::ura4<sup>+</sup></i>	This study
MF78	<i>h<sup>-</sup> atg10::KanMX6 ade6-M210 his7-366 leu1-32 ura4-D18</i>	This study
MF79	<i>h<sup>+</sup> urm1::KanMX6 sty1::his7<sup>+</sup></i>	This study
MF81	<i>h<sup>-</sup> sty1::his7<sup>+</sup> atg8NGFP</i>	This study
MF83	<i>h<sup>-</sup> pREP2(FLAG)urm1:ura4<sup>+</sup> ade6-M210 his7-366 leu1-32 ura4-D18</i>	This study
MF84	<i>h<sup>+</sup> urm1::KanMX6 atf1HA6H:ura4<sup>+</sup> urm1::KanMX6 leu1-32</i>	This study
MF85	<i>h<sup>-</sup> urm1::KanMX6 wis1<sup>DD</sup>(12Myc): ura4<sup>+</sup> ade6-M210 his7-366 leu1-32 ura4-D18</i>	This study
MF88	<i>h<sup>-</sup> wis1<sup>DD</sup>(12Myc): ura4<sup>+</sup> ade6-M210 his7-366 leu1-32 ura4-D18</i>	This study
SW576	<i>h<sup>-</sup></i>	Gift from S. Whitehall

<b>Table 2.2. Oligonucleotide primers used in this study.</b>		
<b>Oligo Name</b>	<b>5'-3' Sequence</b>	<b>Function</b>
Uba1f Uba1r	AACTGCAGAACCAATGCATTGGGGTTGGAAGTGACCA TGC CGGGATCCCGTCAACTTGATACAAATAAATG	Construction of pRIP42PkC- <i>uba1</i>
Uba3f Uba3r	TGCATGCATGCACAGCCTCATCTACCGTTAC CGGGATCCCGTGCATTCTCTCAACTGAACAG	Construction of pRIP42PkC- <i>uba3</i>
Ubc12f Ubc12r	AACTGCAGAACCAATGCATTGGCAGTCATGCTCTAAA TTAGAG CGGGATCCCGTAGCCATGACATTGTCAAAAG	Construction of pRIP42PkC- <i>ubc12</i>
Ubc15f Ubc15r	AACTGCAGAACCAATGCATTGGGCATATCGATTCTTCA CCCAC CGGGATCCCGTAATCATTTCATTGACCTGC	Construction of pRIP42PkC- <i>ubc15</i>
Uba4f Uba4r	AACTGCAGAACCAATGCATTGGCATCACCTCATATTAC TTTCC CGGGATCCCGTATACAGTGGGAAATTTGGGTCG	Construction of pRIP42PkC- <i>uba4</i>
Xpo2f Xpo2r	AACTGCAGAACCAATGCATTGGGCATCGATTGGGAAG CCGATG TCCCCGGGGGATATGTTTTATCGGAAGCGACAG	Construction of pRIP42PkC- <i>xpo2</i>
Atg7f Atg7r	TGCATGCATGCAGGCTGGTCATGAGACATGGTAG CGCGGATCCAAGAATCACACTTTCTTTATCAC	Construction of pRIP42PkC- <i>atg7</i>
Atg3f Atg3r	TGCACTGCAGCAAATACTATAGGACTCCTC CGCGGATCCAGCTTGGATTGTGTAATCAATTTC	Construction of pRIP42PkC- <i>atg3</i>
Atg4f Atg4r	TGCACTGCAGCTACCACCCAGCCTGCTTCC CGCGGATCCAAGATGATCTTCAACATTCTC	Construction of pRIP42PkC- <i>atg4</i>
Atg10f Atg10r	AACTGCAGAACCAATGCATTGGCAAACGGATCAGATC CTCTTACC TCCCCGGGGAGGATTTTTCGGCATCGTCAAC	Construction of pRIP42PkC- <i>atg10</i>
Atg5f Atg5r	AACTGCAGAACCAATGCATTGGCCTCGAGCGCCGGTG CTATGGC CGGGATCCCGTAACTTTCATTAAAACAATATG	Construction of pRIP42PkC- <i>atg5</i>
Uba1chkf	GACTGCACACTTCAAGAGCTTATTG	Verifying the

Uba3chkf	GATGGAGCGTATACATATAC	incorporation of linearised vectors at the correct loci
Ubc12chkf	CGACGATAATTATCCTCATG	
Ubc15chkf	GAAACTACATAGTCTATGCC	
Uba4chkf	CCTACGTTATCTAAACAATTAGC	
Xpo2chkf	GAAGCATTAGCTGCTCGCGCGG	
Atg7chkf	GGCTTCCGACAGTCATATCAAC	
Atg4chkf	CTGGATCCTCATTGTACTCAC	
Atg3chkf	GTTATAAAAGAGGATGAAGGCC	
Atg10chkf	CGCCTGGATTAGGGATTCTCC	
Atg5chkf	GGCATTGGCCTGTTGGCTTAC	
nmtend	GCAGCTTGAATGGGCTTCC	
Ubc15delf	GTGTAATAAAACCTTAGGTAC	Generation of <i>Δubc15</i> strain (MF73)
Ubc15delr	GCTGTTAGTTTTAAATGTC	
Urm1delf	GTCTAATAGTATCGAATACC	Generation of <i>Δurm1</i> strain (MF25)
Urm1delr	GCAAGCTCGGCGAACTCGTAC	
Atg12delf	GTGACAGGAAAGTTGATGC	Generation of <i>Δatg12</i> strain (MF13)
Atg12delr	CTGTCTAAGGCTGACGCTGC	
Atg10delf	GTTGGATAACTTTGTAAACTCC	Generation of <i>Δatg10</i> strain (MF15)
Atg10delr	GTGGAACAGTTGAATGATATG	
KanMX6delchk	CATACAATCGATAGATTGTCTG	Verifying deletion strains
FLAGUrm1oef	GGAATTCCATATGGATTACAAGGACGATGACGACAAG	Construction of pREP2-FLAGUrm1
FLAGUrm1oer	GCTATAAAAGTAGAGTTACTG	
Atg3oef	GGAATTCCATATGGCGCAACGGCTCACATCTGC	Construction of pREP41- <i>atg3</i>
Atg3oer	CGGGATCCCGTCAAGCTTGGATTGTGTAATC	
Atg10oef	GCGATTAATCGCATGAGCTTTAAGTCTCAATTAC	Construction of pREP2- <i>atg10(3Pk)</i> , pREP41- <i>atg10<sup>C131S</sup>(3Pk)</i>
Atg10Pkoer	GAAGATCTTCTTAATGATCAAGACCAAGCAAAG	

Atg10C131Supr	CATGGTATATCCATCCATCTCGTAC	Construction of pREP41- <i>atg10<sup>C131S</sup></i> (3Pk)
Atg10C131Sdnf	AATAATCACGCGTACGAGATGGATG	

## **Chapter 2. Materials and Methods**

### **2.1. Bacterial and Molecular Biology Techniques**

#### **2.1.1. Bacterial Growth Conditions**

XL 1-Blue competent *Escherichia coli* cells (Stratagene) were grown in LB media (1% (w/v) NaCl, 1% (w/v) bactotryptone, 0.5% (w/v) yeast extract). To make solid media, 2% (w/v) Bacto-agar was added.

#### **2.1.2. Bacterial Transformation and Plasmid Isolation**

Plasmids were introduced into bacterial cells by following the manufacturer's instructions (Stratagene). Positive transformants were selected by growth on LB agar (standard LB, plus 2% agar) containing 200µg/ml ampicillin. Transformed cells were cultured overnight in LB Amp (1% (w/v) NaCl, 1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 200µg/ml ampicillin), shaking at 37°C.

Plasmids were isolated using an alkali lysis technique (Birnboim and Doly, 1979). Bacterial cells were harvested, and resuspended in 100µl resuspension solution (50mM glucose, 25mM Tris-HCl pH8.0, 10mM EDTA pH8.0). 200µl lysis solution (0.2mM NaOH, 1% (w/v) SDS) was added, and the mixture incubated at room temperature for up to 5 minutes, before adding 150µl neutralisation solution (3M potassium acetate, 8% (v/v) glacial acetic acid). A biphasic mixture was generated by centrifugation for 10 minutes at 13000g. The supernatant was transferred to a fresh Eppendorf tube, mixed with 1 volume of phenol/chloroform/isoamyl alcohol (25:24:1), and placed in a microfuge for 3 minutes at 13000g. The supernatant, containing plasmid DNA, was

transferred to a fresh tube, and mixed with 2.2 volumes of 100% ethanol. This mixture was incubated at -20°C for 30 minutes and DNA was pelleted by centrifugation for 15 minutes at 13000g. Pellets (containing DNA) were washed with 70% ethanol, and DNA was resuspended in 1x TE buffer.

Plasmids to be used in subsequent ligation reactions were isolated using a GenElute™ miniprep kit (Sigma-Aldrich), and restriction digests performed using Fermentas restriction enzymes (Fermentas, UK), following the manufacturer's instructions.

### **2.1.3. PCR**

When PCR was used to amplify DNA fragments for use in strain construction, an Expand™ HF Polymerase Kit (Roche) was used. Each PCR reaction included 1µl HF DNA polymerase (3.5 units/µl), 10µl HF buffer, 200mM dNTPs (dATP, dCTP, dGTP, dTTP), 1mM primers (synthesised by Eurogentec), ~1µg DNA template and dH<sub>2</sub>O added to give a final volume of 100µl. PCR was performed using a T3 Thermocycler (Biometra), with the conditions outlined below:

Step 1	94°C	30 seconds
Step 2	45-50°C	1 minute
Step 3	68°C	1 minute per kb DNA
Step 4	Repeat steps 1-3 (x35)	
Step 5	68°C	10 minutes
Step 6	4°C	Hold



When PCR was used to amplify DNA fragments for diagnostic purposes, a Pfu<sup>TM</sup> PCR Kit (Finnzymes) was used. Each PCR reaction included 0.5µl Pfu DNA polymerase (2 units/µl), 10µl Pfu buffer, 200mM dNTPs (dATP, dCTP, dGTP, dTTP), 1mM primers (synthesised by Eurogentec), ~1µg DNA template and dH<sub>2</sub>O, added to give a final volume of 50µl. PCR conditions used are outlined below:

Step 1	98°C	30 seconds
Step 2	50-55°C	1 minute
Step 3	72°C	30 seconds per kb DNA
Step 4	Repeat steps 1-3 (x35)	
Step 5	72°C	10 minutes
Step 6	4°C	Hold

#### **2.1.4. PCR Product Analysis and Gel Extraction**

PCR products were analysed using 1% (w/v) agarose gels containing 0.5µg/ml ethidium bromide. Approximate sizes of products were determined using a DNA 1kb Ladder (Fermentas). DNA was extracted from agarose gels using a QIAquick<sup>®</sup> Gel Extraction Kit (QIAGEN), and impurities were removed using a QIAquick<sup>®</sup> Nucleotide Removal Kit (QIAGEN), following the manufacturer's instructions. Restriction digests of PCR products were then performed using Fermentas restriction enzymes (Fermentas, UK), following the manufacturer's instructions.

#### **2.1.5. Plasmid Construction**

#### 2.1.5.1. Integration plasmids

To express proteins tagged at the C-terminus with three copies of the Pk epitope from their chromosomal loci, recombinant pRIP42PkC vectors were constructed (Craven et al., 1998). These plasmids were designated pRIP42PkC-*uba1*, pRIP42PkC-*uba3*, pRIP42PkC-*ubc12*, pRIP42PkC-*ubc15*, pRIP42PkC-*uba4*, pRIP42PkC-*xpo2*, pRIP42PkC-*atg7*, pRIP42PkC-*atg3*, pRIP42PkC-*atg10*, pRIP42PkC-*atg4* and pRIP42PkC-*atg5*.

To generate pRIP42PkC-*uba1*, pRIP42PkC-*ubc12*, pRIP42PkC-*ubc15*, pRIP42PkC-*uba4*, pRIP42PkC-*atg3*, pRIP42PkC-*atg4* and pRIP42PkC-*atg5*, up to 1kb of DNA encoding the C-terminal regions of the respective proteins were amplified from CHP428 (see **Table 2.1**) gDNA by PCR, using oligonucleotide primers outlined in **Table 2.2** (these amplicons did not include stop codons). PCR products were digested with *Bam*HI and *Pst*I restriction enzymes, and ligated with *Bam*HI/*Pst*I-digested pRIP42PkC vector to generate the different constructs. To promote homologous recombination into the yeast genome, these plasmids were linearised prior to transformation. The pRIP42PkC-*uba1*, pRIP42PkC-*ubc15* and pRIP42PkC-*uba4* plasmids were linearised using *Kpn*2I, the pRIP42PkC-*ubc12* and pRIP42PkC-*atg4* plasmids were linearised with *Bgl*II, the pRIP42PkC-*pmk1* plasmid was linearised using *Nde*I, the pRIP42PkC-*atg3* plasmid was linearised with *Bcl*I, and the pRIP42PkC-*atg5* plasmid was linearised using *Bsp*119I.

When *Bam*HI/*Pst*I digests of PCR products were not possible, due to *Bam*HI or *Pst*I sites located near the 3' end of the gene, alternative restriction enzymes were used. To generate the pRIP42PkC-*uba3* and pRIP42PkC-*atg7* plasmids, PCR was performed using oligonucleotide primers listed in **Table 2.2**. PCR products were digested with *Bam*HI and *Nsi*I restriction enzymes and ligated with *Bam*HI/*Pst*I-digested pRIP42PkC vector to create the constructs. The pRIP42PkC-*uba3* plasmid was linearised using *Pst*I, and the pRIP42PkC-*atg7* plasmid was linearised using *Spe*I. To generate the pRIP42PkC-*xpo2* and pRIP42PkC-*atg10* plasmids, PCR was performed using oligonucleotide primers listed in **Table 2.2**. PCR products were digested with *Xma*I and *Pst*I restriction enzymes and ligated with *Xma*I/*Pst*I-digested pRIP42PkC vectors. These











plasmids were linearised using *Bgl*II and *Bsp*119I, respectively.

#### 2.1.5.2. Expression plasmids

The pREP2-*FLAGGurm1* plasmid was constructed by amplifying the *urm1*<sup>+</sup> gene from CHP428 gDNA by PCR, using oligonucleotide primers listed in **Table 2.2**, with the forward primer also including a DNA sequence encoding the FLAG epitope. The PCR product was digested with *Bam*HI and *Nde*I restriction enzymes, and ligated with *Bam*HI/*Nde*I-digested pREP2 vector (Craven et al., 1998). The *arsI* sequence was removed from pREP2-*FLAGGurm1* using the *Eco*RI restriction enzyme. To allow integration of pREP2-*FLAGGurm1* into the genome, the plasmid was linearised using the *Lgu*I restriction enzyme. To generate the pREP41-*atg3* plasmid, the *atg3*<sup>+</sup> gene was amplified by PCR, using the oligonucleotide primers listed in **Table 2.2**. PCR products were digested with *Bam*HI and *Nde*I restriction enzymes, and ligated with *Bam*HI/*Nde*I-digested pREP41 vector (Craven et al., 1998). To generate pREP2-*atg10(3Pk)*, an ORF encoding Pk epitope-tagged Atg10 was amplified from MF11 gDNA by PCR, using oligonucleotide primers listed in **Table 2.2**. The PCR product was digested using *Bgl*III and *Vsp*I restriction enzymes before being ligated with *Bgl*III/*Vsp*I-digested pREP2 vector. To generate pREP41-*atg10*<sup>C131S</sup>(*3Pk*), overlapping PCR was performed (see Section 5.3.3), using the oligonucleotide primers listed in **Table 2.2** and MF11 gDNA as a template for reaction.

#### 2.1.5.3. Plasmid sequencing

All recombinant plasmids were sequenced by GATC Biotech.



## 2.2. *S. pombe* Techniques

### 2.2.1. Yeast Growth Conditions and Measurements

The *S. pombe* strains used in this study are listed in **Table 2.1**. Yeast strains were cultured in YE5S rich media (0.5% (w/v) yeast extract, 3% (w/v) glucose, 225mg/L adenine hemisulphate, L-histidine, uracil, L-lysine monochloride and L-leucine) or EMM media (3g/L potassium hydrogen phthalate, 2.2g/L Na<sub>2</sub>HPO<sub>4</sub>, 5g/L NH<sub>4</sub>Cl, 2% (w/v) glucose, 20ml/L salt solution [from 50x stock: 52.5g/L MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.725g/L CaCl<sub>2</sub>·2H<sub>2</sub>O, 50g/L KCl, 2g/L Na<sub>2</sub>SO<sub>4</sub>], 1ml/L vitamins solution [from 1000x stock: 1g/L pantothenic acid, 10g/L nicotinic acid, 10g/L inositol, 10mg/L biotin], 0.1ml/L minerals [from 10000x stock: 5g/L boric acid, 4g/L MnSO<sub>4</sub>, 4g/L ZnSO<sub>4</sub>·7H<sub>2</sub>O, 2g/L FeCl<sub>2</sub>·6H<sub>2</sub>O, 0.4g/L molybdic acid, 1g/L KI, 0.4g/L CuSO<sub>4</sub>·5H<sub>2</sub>O, 10g/L citric acid]) with adenine, histidine, leucine and uracil supplements, as outlined previously (Alfa et al., 1993; Moreno et al., 1991). EMM minimal glutamate media containing 1g/L sodium glutamate in place of NH<sub>4</sub>Cl (EMM<sup>1</sup>/<sub>2</sub>G) was used for genetic crosses via induction of conjugation and sporulation. EMM lacking NH<sub>4</sub>Cl, adenine, histidine, leucine or uracil (EMM-N) was used for nitrogen starvation of cells (Kohda et al., 2007; Watanabe et al., 1988). To make solid media, 2% (w/v) Bacto-agar was added. Strains expressing the *KanMX6* cassette were selected by growth on solid YE5S media containing 125µg/ml G-418 disulphate (Melford Laboratories Ltd.). Yeast cell growth was monitored using a UV-1601 Shimadzu Spectrophotometer. Cell density was measured at OD<sub>595nm</sub> (where absorbance 0.5 = 1x10<sup>7</sup> cells/ml).

### 2.2.2. Cell Growth and Survival Assays

Yeast cultures were grown to mid-log phase (OD<sub>595nm</sub> of 0.25-0.5) in YE5S media, treated with the indicated stress agent, and incubated at 30°C.

To monitor cell growth, cells were counted using a CASY<sup>®</sup> Model TT Cell Counter and Analyser System (Schärfe System GmbH). Growth curves were plotted by calculating fold increase in cell numbers at the different time points, relative to cell numbers at time 0.

To monitor cell survival, cells were treated with a range of concentrations of stress agent, or untreated. Cells were counted at various time points, and serial dilutions performed such that 1ml of YE5S contained ~1000 cells. 100µl of this dilution were plated onto YE5S solid media, thus transferring ~100 cells to each plate. Plates were incubated at 30°C for 2-3 days, and colonies were counted. Survival rates were calculated by dividing the number of viable colonies on plates of stressed cells by the number of viable colonies on plates of untreated cells, which was taken to represent 100% survival.

### **2.2.3. Genetic Crosses**

#### *2.2.3.1. Mating*

To perform genetic crosses, *S. pombe* strains to be mated were streaked out on YE5S solid media, and incubated at 30°C overnight. To mate two strains, cells were mixed together on EMM<sup>1</sup>/<sub>2</sub>G media in 5µl dH<sub>2</sub>O, and incubated at 25°C for 2-3 days to allow tetrad formation.

#### *2.2.3.2. Tetrad dissection*

Following tetrad formation, a sample of cells was resuspended in 0.5ml dH<sub>2</sub>O, and 15µl pipetted in a line onto YE5S solid media. Tetrads were isolated using a Singer MSM dissection microscope and incubated at 37°C until asci fragmented. Spores were then

separated using the dissector, and plates were incubated at 30°C to allow colony development. The genotypes of the resulting colonies were determined by replica plating onto appropriate selective media, and by PCR analyses.

#### **2.2.4. Induction of Autophagy**

Autophagy was induced in *S. pombe* cells by nitrogen starvation, as previously described (Kohda et al., 2007). Cultures were grown to mid-log phase (OD<sub>595nm</sub> 0.25-0.5) in EMM, split into two equal volumes, and cells were harvested by centrifugation. To induce autophagy in the first culture (lacking a nitrogen source; N-), cells were washed three times in 10ml EMM-N, and resuspended in EMM-N to the same volume as the original culture. For the second culture (including a nitrogen source; N+), cells were resuspended in EMM, again to the same volume as the original culture. Both of these cultures were then incubated at 30°C for 20 hours. Cells were then analysed by fluorescence microscopy (see Section 2.2.11). Cell extracts were also prepared (see Section 2.2.8.1) and analysed by western blotting (see Section 2.2.10) using anti-GFP antibodies (Invitrogen A6455).

#### **2.2.5. Sensitivity Tests**

All sensitivity tests were performed using *S. pombe* cells growing at mid-log phase (OD<sub>595nm</sub> of 0.25-0.5) in YE5S media, unless otherwise stated. Cells were diluted in YE5S to give an OD<sub>595nm</sub> of 0.25, in a final volume of 1ml. Serial 1:10 dilutions were performed, again using YE5S. Finally, equivalent cell volumes were spotted onto solid media containing different concentrations of stress agents. YE5S plates were incubated at 30°C for 3 days before scanning.

Oxidative stress sensitivity was analysed using H<sub>2</sub>O<sub>2</sub>, the GSH-depleting agent diamide, and the alkyl hydroperoxide *t*-BOOH. Osmotic stress sensitivity was analysed using KCl. Cell cycle control was investigated using the ribonucleotide reductase inhibitor

HU, which inhibits DNA replication, and the microtubule depolymerising agent TBZ, which inhibits spindle formation during mitosis. To investigate heat sensitivity, plates were incubated at 37°C for 3 days.

#### **2.2.6. gDNA Extraction**

*S. pombe* cells were collected from agar plates and transferred to screw-cap ribolyser tubes containing 1ml dH<sub>2</sub>O. Cells were harvested by centrifugation at 9000g for 1 minute, and washed in dH<sub>2</sub>O. The dH<sub>2</sub>O was removed, and cell pellets were resuspended in 200µl chromosomal DNA breakage buffer (10mM Tris-HCl pH8, 1mM EDTA pH8, 100mM NaCl, 1% (w/v) SDS, 2% (v/v) Triton X-100). 200µl glass beads (400-600 microns) and 200µl phenol/chloroform/isoamyl alcohol (25:24:1) were also added to the tubes. Cells were lysed using a Biospec mini bead-beater for 30 seconds. A further 500µl of DNA breakage buffer were added, and the tubes vortexed briefly. A biphasic mixture was generated by centrifugation at 9000g for 5 minutes. The upper phase (containing DNA) was transferred to a fresh Eppendorf tube.  $\frac{1}{10}$  volume of 3M sodium acetate and 2 volumes of 100% ethanol were added to promote DNA precipitation. Tubes were incubated at -20°C for 30 minutes and DNA pelleted by centrifugation at 13000g for 15 minutes. The supernatant was removed, and pellets of precipitated DNA were washed with 70% ethanol. After drying in air, gDNA pellets were resuspended in 100µl dH<sub>2</sub>O.

#### **2.2.7. LiAc Transformation**

DNA was introduced into yeast cells using a LiAc procedure (Moreno et al., 1991). Stationary phase cultures were diluted to OD<sub>595nm</sub> ~0.15 in 100ml YE5S and incubated at 30°C until they reached mid-log phase of exponential growth (OD<sub>595nm</sub> of 0.25-0.5). Cells were harvested by centrifugation at 7000g for 30 seconds. Cell pellets were washed with water and 1x LiAc/TE (0.1M LiAc pH7.5, 10mM Tris-HCl pH7.5, 1mM EDTA pH8), and resuspended in 1ml 1x LiAc/TE. Transforming DNA (0.1-1µg) and

ssDNA (to a final concentration of 100µg/ml) were added to 100µl of the resuspended cells, and the mixtures incubated at room temperature for 10 minutes. 260µl LiAc/TE/PEG (0.1M LiAc pH7.5, 10mM Tris-HCl pH7.5, 1mM EDTA pH8, 50% (w/v) PEG 4000) were added, and the mixtures were incubated at 30°C for 30-60 minutes. 43µl DMSO were added (giving a final concentration of 10% (v/v) DMSO), and cells were incubated at 42°C for 5 minutes to heat shock. Cells were harvested by centrifugation, washed with water, and resuspended in 250µl dH<sub>2</sub>O. Finally, cells were plated onto selective media, and plates were incubated at 30°C for 3-5 days.

## **2.2.8. Protein Preparation**

### *2.2.8.1. Whole cell extracts*

To prepare whole cell extracts for western blotting, mid-log phase (OD<sub>595nm</sub> of 0.25-0.5) growing *S. pombe* cultures were harvested by centrifugation at 3000g for 2 minutes. Cells were resuspended in 1ml dH<sub>2</sub>O and transferred to Eppendorf tubes. Cells were pelleted by centrifugation at 9000g for 1 minute, and the supernatant removed. Cell pellets were snap-frozen in liquid nitrogen, and allowed to thaw at room temperature. Cells were resuspended in 200µl ice-cold *S. pombe* lysis buffer (50mM Tris-HCl pH7.5, 150mM NaCl, 0.5% (v/v) Nonidet P-40, 10mM imidazole), including protease inhibitors (0.1% (w/v) leupeptin, 0.1% (w/v) pepstatin A, 1% (v/v) aprotinin, 1% (w/v) PMSF) and phosphatase inhibitors (0.2% (w/v) Na<sub>3</sub>VO<sub>4</sub>, 5% (w/v) NaF), and added to screw-cap ribolyser tubes containing 1ml of ice-cold glass beads. When analysing Urm1 conjugation, *S. pombe* lysis buffer also included 20mM NEM. Cells were lysed using a bead beater for 20-30 seconds twice, with a 1-2 minute cooling period in between. A further 100-200µl *S. pombe* lysis buffer were then added to the tubes. A 19-gauge needle was used to pierce the bottom of the ribolyser tubes, which were then placed inside pre-chilled Eppendorf tubes contained inside 50ml Falcon tubes. To wash protein samples into the Eppendorf tubes, the Falcon tubes were spun at 2000g for 1 minute in a pre-cooled centrifuge. Soluble and insoluble protein samples were then separated by

centrifugation at 13000g for 10 minutes in a pre-cooled centrifuge. Finally, protein was transferred to fresh Eppendorf tubes, and stored at -80°C. When investigating protein phosphorylation, extracts were prepared using lysis buffer lacking phosphatase inhibitors, and treated with Lambda protein phosphatase (New England Biolabs), following the manufacturer's instructions. Relative protein concentrations were determined using Coomassie Protein Assay Reagent (Thermo Scientific), following the manufacturer's instructions.

#### 2.2.8.2. TCA protein preparation

To investigate the redox status of cysteine residues, cell lysates were prepared by TCA lysis using a technique described previously (Delaunay et al., 2000). To modify reduced cysteines with AMS, protein pellets were resuspended in acid lysis buffer containing 10µg/ml PMSF and 12.5mg/ml AMS (Sigma). To treat extracts with TCEP, lysis buffer also contained 100mM TCEP. Relative protein concentrations were determined using a Pierce<sup>®</sup> BCA Protein Assay Kit (Thermo Scientific), following the manufacturer's instructions.

#### 2.2.8.3. MAPK phosphorylation assays

To retain the phosphorylation state of MAPKs during protein preparation, the procedure outlined in Section 2.2.8.1 was modified. Mid-log phase (OD<sub>595nm</sub> of 0.25-0.5) growing *S. pombe* cultures were harvested by centrifugation at 3000g for 2 minutes, using a pre-cooled centrifuge and 50ml Falcon tubes containing 25ml ice. The supernatant was removed, and cell pellets quickly snap-frozen in liquid nitrogen. Cell pellets were thawed in 1ml ice-cold *S. pombe* lysis buffer, also containing 0.7% (v/v) β-mercaptoethanol, and transferred to pre-chilled Eppendorf tubes. Cells were pelleted by centrifugation at 7000g for 1 minute. The supernatant was removed using a vacuum, and cells were resuspended in 250µl ice-cold *S. pombe* lysis buffer containing 0.7% (v/v) β-mercaptoethanol. As described in Section 2.2.8.1, cells were added to screw-cap

ribolyser tubes containing 1ml of ice-cold glass beads, and lysed using a bead beater. Cell lysates were transferred to pre-chilled Eppendorf tubes, again as described in Section 2.2.8.1. Protein concentrations in cell lysates were determined using Coomassie Protein Assay Reagent (Thermo Scientific), and samples of these lysates were added to SDS loading dye (from 4x stock: 100mM Tris-HCl pH6.8, 200mM  $\beta$ -mercaptoethanol, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol). Protein samples were stored at -20°C.

### **2.2.9. Western Blotting**

To analyse protein extracts by western blotting, proteins were separated by SDS polyacrylamide gel electrophoresis, using SDS gel recipes outlined previously (Laemmli, 1970). Proteins were transferred from the gel to a Protran<sup>®</sup> nitrocellulose membrane (Schleicher and Schuell Biosciences) using a Helena Biosciences transfer kit, following the manufacturer's instructions. After transfer was completed, the nitrocellulose membrane was removed, and washed with 10% (w/v) BSA – made up in 1x TBS-T (10x TBS [10mM Tris-HCl pH8, 150mM NaCl], 0.1% (v/v) Tween20) – for 45 minutes to block non-specific protein binding sites. The membrane was washed in 1x TBS-T for 5 minutes, then incubated with primary antibody (diluted 1:1000 in 5% (w/v) BSA made up in 1x TBS-T) overnight at 4°C. The antibody was removed, and the membrane washed in 1x TBS-T three times for 5-10 minutes. The membrane was then incubated with secondary antibody (horse radish peroxidase [HRP]-conjugated anti-mouse or anti-rabbit IgG [Sigma], diluted 1:2000 in 5% (w/v) BSA made up in 1x TBS-T) for 1 hour at room temperature. The antibody was removed, and the membrane washed in 1x TBS-T three times for 5-10 minutes. Finally, the membrane was developed using the ECL<sup>™</sup> detection system (Amersham Pharmacia Biotech).

To reprobe with a different antibody, nitrocellulose membranes were stripped by incubating at 50°C in SDS stripping solution (2% SDS, 62.5mM Tris-HCl pH6.7, 100mM  $\beta$ -mercaptoethanol) for 30 minutes. A mouse anti-TAT1 antibody that targets  $\beta$ -tubulin (Cancer Research UK; diluted 1:1000 in 5% (w/v) BSA made up in 1x TBS-T) or a rabbit antibody that targets Sty1 (polyclonal anti-Sty1 IgG; diluted 1:1000 in 5%

(w/v) BSA made up in 1x TBS-T) (Day and Veal, 2010) were used with anti-mouse or anti-rabbit IgG secondary antibodies as above, to determine protein loading.

## **2.2.10. Fluorescence Microscopy**

### *2.2.10.1. Indirect immunofluorescence*

To investigate protein localisation, 10ml of mid-log phase ( $OD_{595nm}$  of 0.25-0.5) growing cultures were fixed in 3.7% (w/v) paraformaldehyde, and incubated at room temperature for 30 minutes. Cells were harvested by centrifugation at 6000g for 1 minute, and washed in 1x PEM (10mM PIPES pH6.9, 1mM EDTA pH8, 1mM  $MgSO_4$ ) three times. To break down the yeast cell wall, cells were resuspended in PEMS (1x PEM, 1.2M sorbitol) containing 0.5 $\mu$ g/ml zymolase 20-T (ICN Biomedicals), and incubated at 37°C for 70 minutes. Cells were pelleted by centrifugation, resuspended in PEMS containing 1% (v/v) Triton X-100, and incubated at room temperature for 1 minute. Cells were washed three times with 1x PEM. To block non-specific antibody binding sites, cells were resuspended in PEMBAL (1x PEM, 1% (w/v) BSA, 0.1% (w/v) sodium azide, 0.1M L-lysine monohydrochloride), and incubated at room temperature for 30 minutes. Cells were harvested by centrifugation, resuspended in 200 $\mu$ l primary antibody (diluted 1:1000 in PEMBAL), and incubated overnight at room temperature. Primary antibody was removed, and cells were washed three times in PEMBAL. Following centrifugation, cells were resuspended in 200 $\mu$ l secondary antibody (Alexa Fluor® FITC-conjugated anti-mouse or anti-rabbit antibody [Invitrogen], diluted 1:200 in PEMBAL) and incubated for 90 minutes at room temperature. Finally, cells were washed three times in PEMBAL, and resuspended in 0.5ml PEMBAL for use in microscopy.

### *2.2.10.2. Microscopy*



10-20 $\mu$ l cells were spread onto microscope slides coated in poly-L-lysine, and allowed to dry in air. Slides were submerged in -20°C methanol for 6 minutes, then in -20°C acetone for 30 seconds, and allowed to dry. Vectashield mounting medium containing DAPI (Vector Laboratories) was used to mount cover slips onto slides. Cells were examined using a Zeiss Axiovision microscope with a 63x oil immersion objective and Axiovision imaging system. DAPI fluorescence was measured by exciting at 365nm and detecting at 465nm; FITC fluorescence was measured by exciting at 450-490nm and detecting at 510-550nm.

## Extra References

- Alfa, C.E., Gallagher, I.M., and Hyams, J.S. (1993). Antigen localization in fission yeast. *Methods in cell biology* 37, 201-222.
- Birnboim, H.C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic acids research* 7, 1513-1523.
- Craven, R.A., Griffiths, D.J., Sheldrick, K.S., Randall, R.E., Hagan, I.M., and Carr, A.M. (1998). Vectors for the expression of tagged proteins in *Schizosaccharomyces pombe*. *Gene* 221, 59-68.
- Delaunay, A., Isnard, A.D., and Toledano, M.B. (2000). H<sub>2</sub>O<sub>2</sub> sensing through oxidation of the Yap1 transcription factor. *The EMBO journal* 19, 5157-5166.
- Kohda, T.A., Tanaka, K., Konomi, M., Sato, M., Osumi, M., and Yamamoto, M. (2007). Fission yeast autophagy induced by nitrogen starvation generates a nitrogen source that drives adaptation processes. *Genes to cells : devoted to molecular & cellular mechanisms* 12, 155-170.
- Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.
- Moreno, S., Klar, A., and Nurse, P. (1991). Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods in enzymology* 194, 795-823.
- Watanabe, Y., Lino, Y., Furuhashi, K., Shimoda, C., and Yamamoto, M. (1988). The *S.pombe* mei2 gene encoding a crucial molecule for commitment to meiosis is under the regulation of cAMP. *The EMBO journal* 7, 761-767.

## **Chapter 3. Redox regulation of enzymes functioning in the ubiquitination and neddylation pathways.**

### **3.1. Introduction**

Ubiquitin and Ubls become attached to substrate proteins as part of highly conserved conjugation pathways in eukaryotes. Ubiquitin/Ubl-activating (E1) and -conjugating (E2) enzymes form transient thioester bonds to specific modifiers, before promoting their conjugation to specific downstream targets. This process also involves ligase (E3) enzymes to direct conjugation to specific proteins. Post-translational modification of proteins by ubiquitin/Ubl conjugation can influence protein stability, localisation, activity and protein-protein interactions in a manner analogous to phosphorylation, allowing regulation of numerous cellular processes (Herrmann et al., 2007; Hochstrasser, 2000, 2009; Kerscher et al., 2006; Welchman et al., 2005). Importantly, E1s and E2s utilise catalytic cysteine residues that facilitate thioester formation to ubiquitin/Ubls.

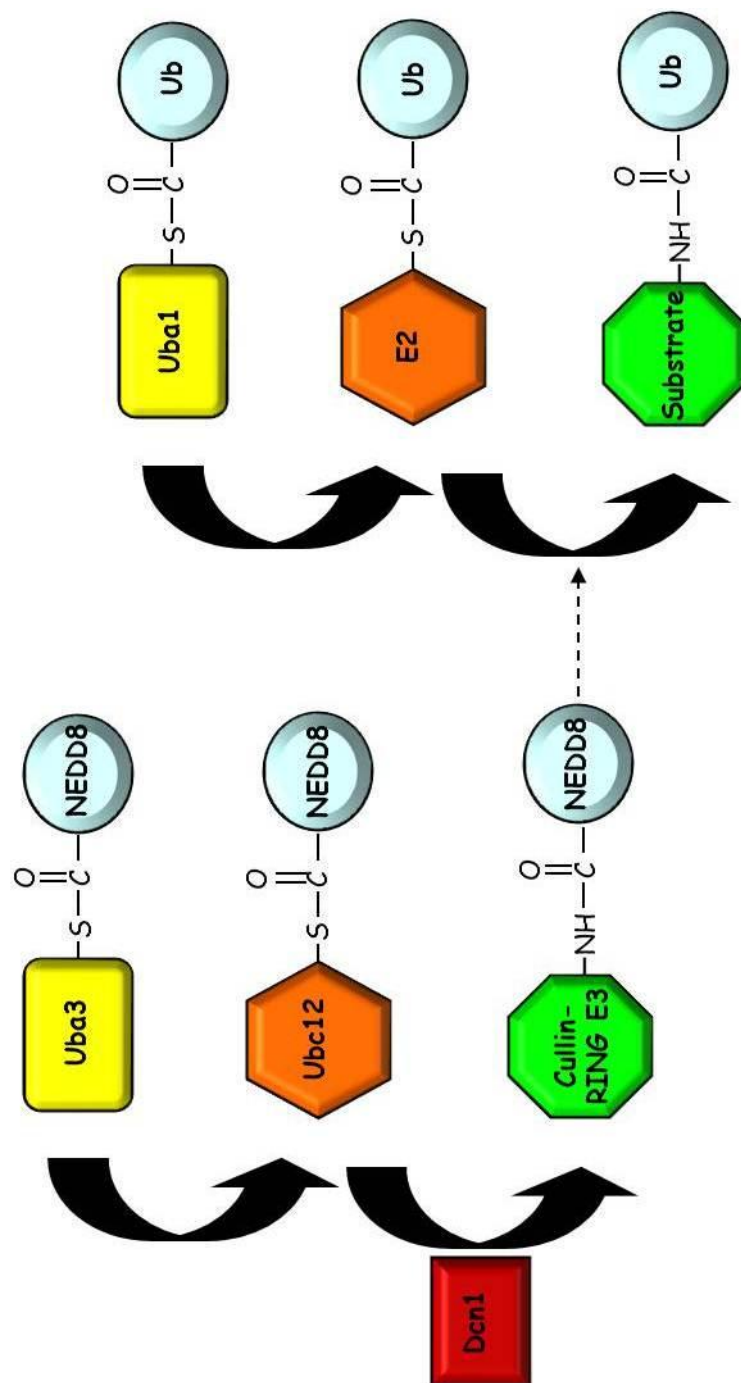
Recent work has demonstrated that SUMOylation and neddylation can be inhibited during oxidative stress in mammalian cells. Oxidation of the catalytic cysteine residue of the SUMO-specific E1, Uba2, and/or the catalytic cysteine of the E2, Ubc9, causes the formation of an inhibitory disulfide complex involving the two enzymes, thus preventing SUMOylation (Bossis and Melchior, 2006). Similarly, neddylation is inhibited by oxidation of the catalytic cysteine of the NEDD8-specific E2, Ubc12. Although Ubc12 forms disulfide complexes following cysteine oxidation, these complexes do not involve the E1, Uba3 (Kumar et al., 2007). However, the downstream consequences of inhibiting Ubl conjugation during oxidative stress are largely unknown. Furthermore, while redox-regulation of Ubl conjugation has been observed in mammalian cells, it is unclear whether such mechanisms operate in all eukaryotes.

Recent research in our lab has demonstrated that the ubiquitin-specific E1, Uba1, and an E2, Cdc34, form inhibitory disulfide complexes during oxidative stress in budding yeast (our unpublished data). This results in the accumulation of a Cdk1, Sic1, and causes transient cell cycle arrest in G1 phase. However, it is unclear whether this mechanism is conserved in other eukaryotes. Consequently, an objective of this project was to investigate redox regulation of ubiquitin/Ubl conjugation pathways in the model organism *S. pombe*, and identify which regulatory mechanisms are conserved and which are organism-specific. A second objective was to investigate whether redox regulation of these conjugation pathways impacted upon the oxidative stress response mechanisms of *S. pombe*. Finally, a third objective was to identify any structural properties of E1s or E2s that may render their catalytic cysteines sensitive to oxidation.

### **3.2. Investigation of the redox-sensitivities of Uba1, Uba3 and Ubc12 during oxidative stress.**

#### **3.2.1. Pk epitope-tagging of Uba1, Uba3 and Ubc12 at C-termini.**

As outlined in Section 1.1, ubiquitination and neddylation are interrelated processes (**Fig. 3.1**). Previous investigations have shown that ubiquitination and neddylation can be redox-regulated in other organisms. Hence, it was possible that this could also be the case in *S. pombe*, and any redox regulation may contribute to oxidative stress responses. To investigate the redox-sensitivities of Uba1, Uba3 and Ubc12 in *S. pombe*, it was essential to express epitope-tagged versions of these proteins. BLAST searches were used to identify *S. pombe* homologues of Uba1, Uba3 and Ubc12, using *S. cerevisiae* amino acid sequences. Genes encoding these enzymes were designated *uba1*<sup>+</sup>, *uba3*<sup>+</sup> and *ubc12*<sup>+</sup>, respectively. The *uba1*<sup>+</sup> gene is located on chromosome II, and encodes a protein with a predicted M<sub>r</sub> of ~110kDa. The gene encoding Uba1 has previously been identified in a genetic screen for mutants with defective nucleocytoplasmic transport of mRNA and proteins, and is essential for viability (Azad et al., 1997). The *uba3*<sup>+</sup> gene is found on chromosome I and encodes the catalytic subunit of the dimeric NEDD8 E1.

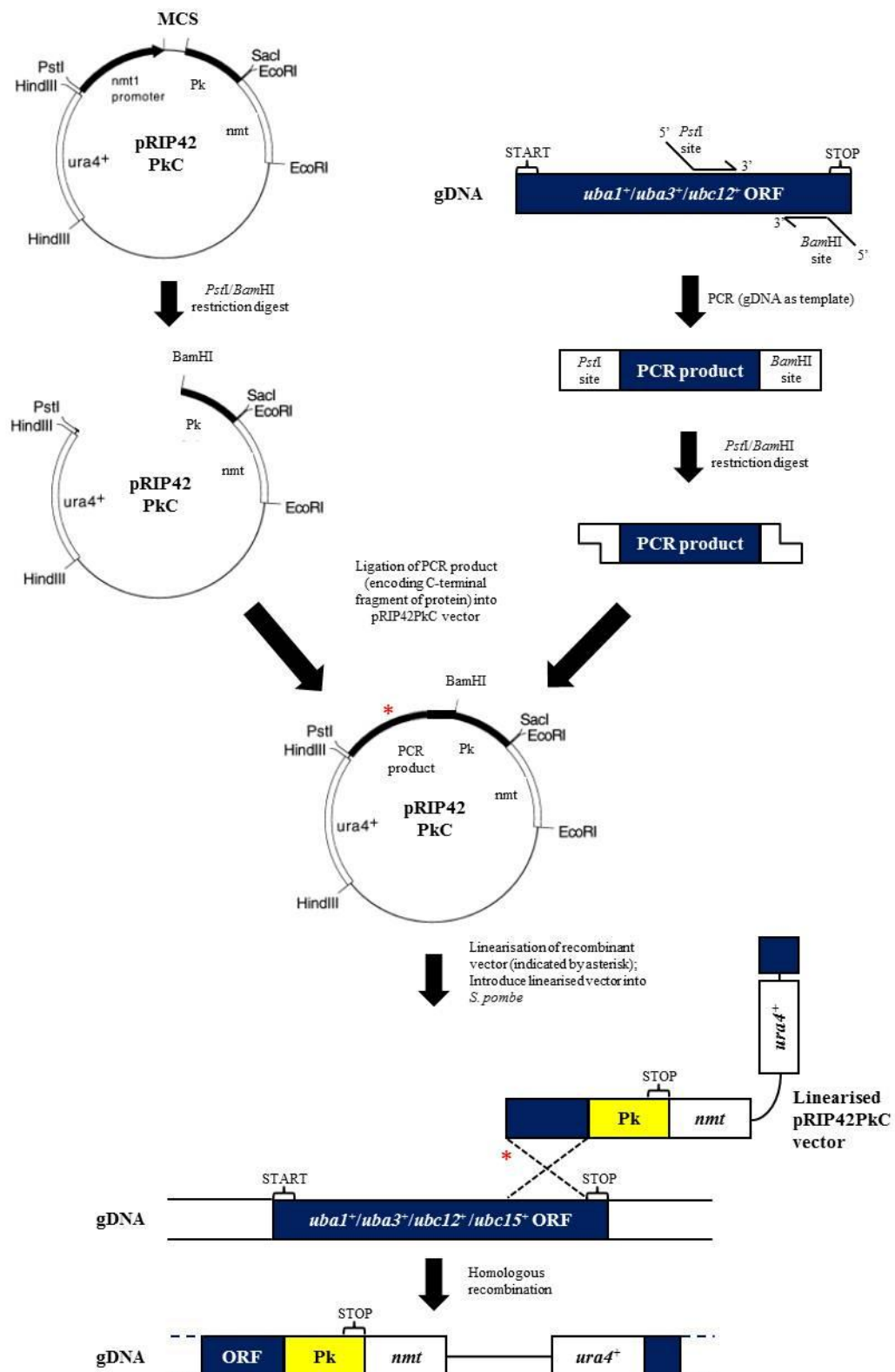


**Figure 3.1. The ubiquitination of many proteins is dependent on neddylation of cullin-RING E3 ligases.** NEDD8 is conjugated to the cullin subunit of cullin-RING E3 ligases via the E1, Uba3, and the E2, Ubc12, as well as NEDD8-specific E3 ligases, such as Dcn1. Cullin neddylation promotes the recruitment of ubiquitin-loaded E2s to the cullin-RING E3. Ubiquitin is subsequently conjugated to downstream substrates due to the concerted actions of the ubiquitin-loaded E2 and a neddylation cullin-RING E3 ligase.

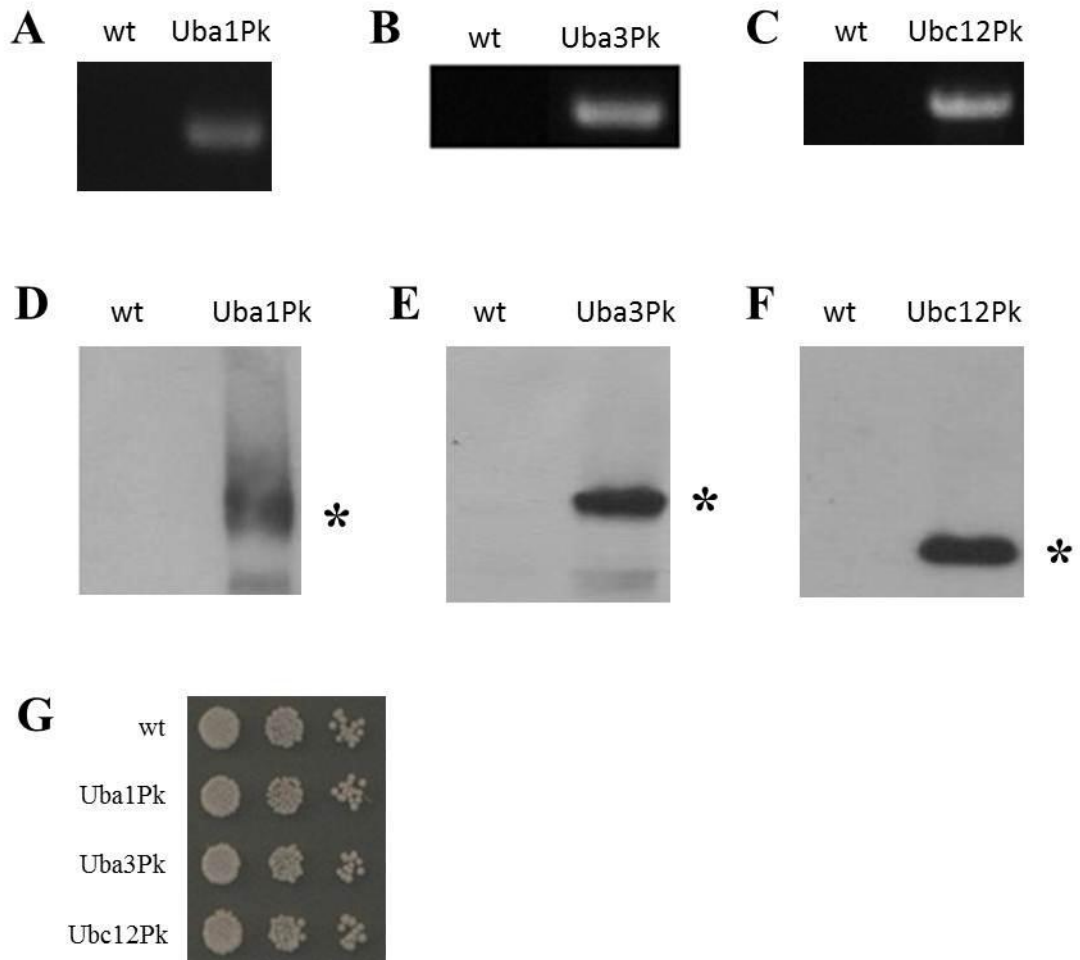
The protein has a predicted  $M_r$  of ~50kDa. Finally, *ubc12*<sup>+</sup> is located on chromosome III, and encodes the Ubc12 protein, which has a predicted  $M_r$  of ~20kDa. Another group has previously shown that these genes encode Uba3 and Ubc12, and that both genes are essential for viability in *S. pombe* (Osaka et al., 2000).

Previous investigations have used western blotting to analyse epitope-tagged E1s and E2s and detect disulfide complex formation during oxidative stress (Bossis and Melchior, 2006; Kumar et al., 2007). *S. pombe* proteins are often tagged with Pk epitopes to allow their investigation using techniques such as western blotting and indirect immunofluorescence. Furthermore, anti-Pk antibodies are commercially available, and bind Pk epitopes with high specificity (Craven et al., 1998). Therefore, the strategy outlined in **Fig. 3.2** was used to express Uba1, Uba3 and Ubc12 tagged with three copies of the Pk epitope at their C-termini, and expressed from their normal chromosomal loci. First, three integration tagging plasmids – pRIP42PkC-*uba1*, pRIP42PkC-*uba3* and pRIP42PkC-*ubc12* – were constructed (see Section 2.1.5.1 and **Fig. 3.2**), linearised, and introduced individually into CHP428 wild-type cells to allow integration into the genome via homologous recombination. Integration of plasmids at the specific gene loci was confirmed by PCR (**Fig. 3.3A-C**), and the expression of Pk epitope-tagged proteins was confirmed by western blotting (**Fig. 3.3D-F**). The estimated  $M_r$  values of the tagged proteins were as expected (Uba1Pk, ~110kDa; Uba3Pk, ~50kDa, Ubc12Pk, ~25kDa).

It was possible that tagging these enzymes could affect their activity, interactions or stability. As *uba1*<sup>+</sup>, *uba3*<sup>+</sup> and *ubc12*<sup>+</sup> are all essential genes in *S. pombe*, any effect of Pk-tagging on enzyme function may result in growth defects or loss of viability. Therefore, the growth of strains expressing Pk-tagged Uba1, Uba3 and Ubc12 was investigated using solid YE5S medium. Pk-tagging of these enzymes did not appear to affect cell growth or viability, when compared to a wild-type strain (**Fig 3.3G**). Pk epitopes at their C-termini. Taken together, these data show that Uba1Pk, Uba3Pk and Ubc12Pk are all stably-expressed, and that Pk-tagging does not appear to affect the function of these enzymes.



**Figure 3.2. Strategy used to express proteins tagged with Pk epitopes at their C-termini from their normal locus, via the introduction of recombinant vectors** (Adapted from Craven *et al.* 1998, Fig. 5). Oligonucleotide primers were designed to amplify a region of the gene encoding a C-terminal fragment of the protein (up to but not including the STOP codon), flanked by *Pst*I and *Bam*HI restriction sites (see Table 2.2). After digestion with *Pst*I and *Bam*HI restriction enzymes, the PCR product was ligated with *Pst*I/*Bam*HI-digested pRIP42PkC vector. When this was not possible, different restriction sites were used (see section 2.1.5.1). An additional restriction enzyme was used to linearise the recombinant vector at the point indicated by the red asterisk. Linearised, recombinant vectors were introduced into *S. pombe* cells, allowing incorporation of the recombinant pRIP42PkC plasmid at the desired locus by homologous recombination. Potential integrants were selected by growth on solid media lacking uracil. Pk-tagging of proteins was confirmed by SDS-PAGE and western blotting, using anti-Pk antibodies.



**Figure 3.3. Pk epitope-tagging of Uba1, Uba3 and Ubc12 enzymes at their C-termini has no effect on viability.**

**A-C.** To confirm integration of pRIP42PkC, gDNA was extracted from wild-type (wt; CHP428) cells and from cells transformed with linearised, recombinant pRIP42PkC to tag (A) Uba1 (MF32), (B) Uba3 (MF34) and (C) Ubc12 (MF36). Integration of plasmids at the correct loci was confirmed by PCR, using the forward primers (A) Uba1chkf, (B) Uba3chkf and (C) Ubc12chkf, which bind to sites within the respective ORFs, and the reverse primer nmtend, which binds within the nmt sequence of pRIP42PkC (see Table 2.2). PCR products were analysed by agarose gel electrophoresis, and PCR products of (A) ~600bp, (B) ~500bp and (C) ~800bp were detected as confirmation of strain construction. **D-F.** Protein extracts from strains expressing (D) Uba1Pk, (E) Uba3Pk and (F) Ubc12Pk were prepared and analysed by SDS-PAGE and western blotting under reducing conditions. Extracts from wild-type (wt; CHP428) cells were used as controls. Uba1Pk (~110kDa), Uba3Pk (~50kDa) and Ubc12 (~25kDa) are indicated by asterisks. **G.** Ten-fold serial dilutions of mid-log phase wild-type (wt; CHP428), Uba1Pk (MF32), Uba3Pk (MF34) and Ubc12Pk (MF36) cells were spotted onto solid YE5S media. Plates were incubated at 30°C for three days.

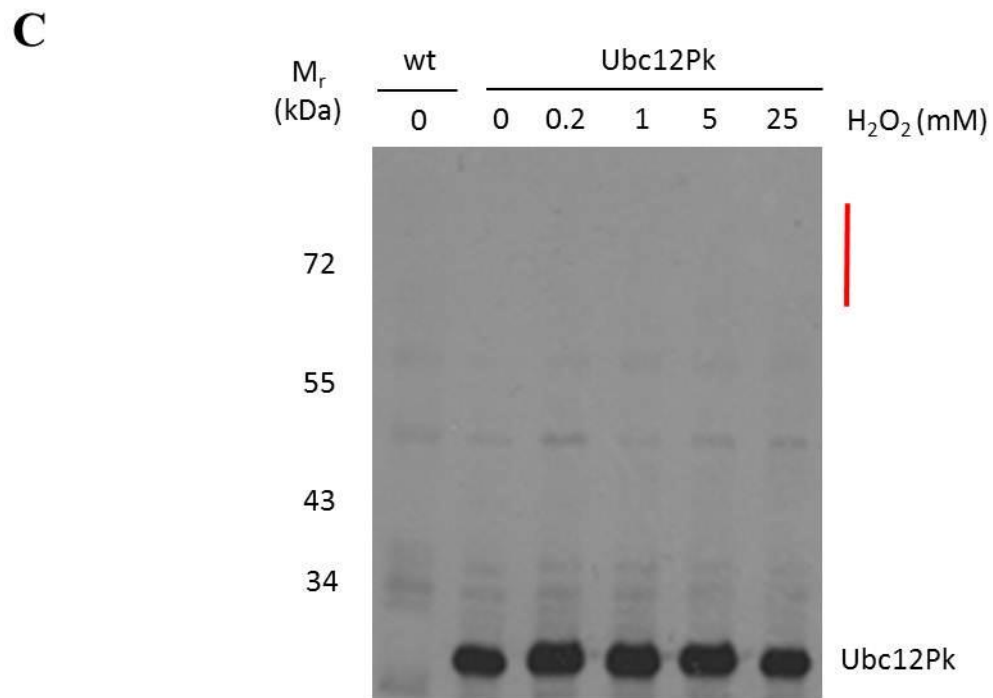
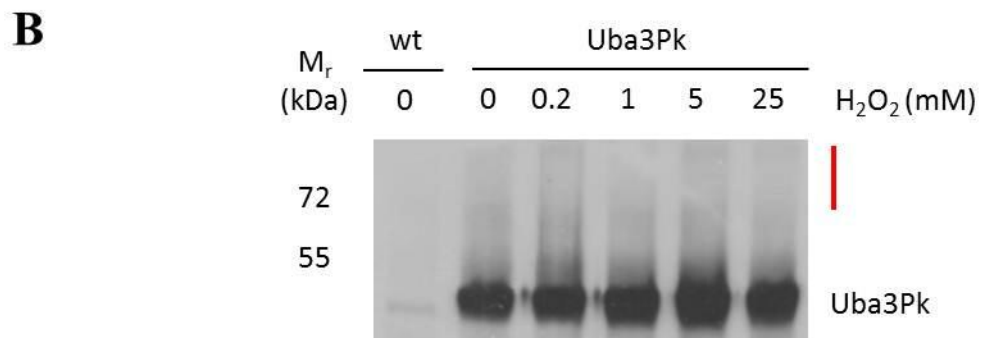
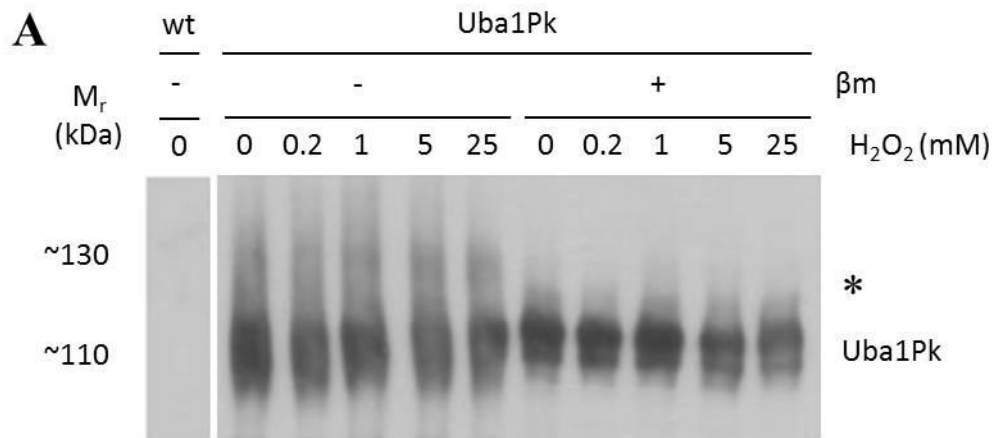


Furthermore, when cells expressing the tagged proteins were analysed by microscopy, no obvious phenotypic changes were observed (data not shown), suggesting that the functions of Uba1, Uba3 and Ubc12 are unaffected when these enzymes are tagged.

### **3.2.2. Uba1, but not Uba3 or Ubc12, forms HMW complexes in response to oxidative stress.**

As outlined previously, Uba1 forms an inhibitory disulfide with a specific E2 in *S. cerevisiae* (our unpublished data), and Ubc12 forms HMW disulfide complexes in human cells (Kumar et al., 2007). Therefore, it is possible that these enzymes are also redox-regulated via catalytic cysteines in evolutionarily divergent organisms such as *S. pombe*. To investigate whether H<sub>2</sub>O<sub>2</sub> treatment causes the formation of HMW complexes involving Uba1, Uba3 and Ubc12, cells expressing Uba1Pk, Uba3Pk or Ubc12Pk were exposed to a range of H<sub>2</sub>O<sub>2</sub> concentrations, and cell extracts prepared by acid lysis. Non-reducing SDS-PAGE and western blotting were used for protein analysis. Unexpectedly, Uba1 was found to form HMW complexes under homeostatic conditions (**Fig. 3.4A**). The nature of these complexes is unknown, but they may be Uba1-ubiquitin thioester intermediates or associations with other proteins. Indeed, the reduced mobility of these complexes through SDS-PAGE gels is consistent with E1-E2 complexes, as an E2 would be expected to add ~20kDa to the M<sub>r</sub> of Uba1. Interestingly, high levels of H<sub>2</sub>O<sub>2</sub> ( $\geq 1\text{mM H}_2\text{O}_2$ ) cause a change in the nature of these complexes, as reflected in altered mobility (**Fig. 3.4A**). Significantly, all these HMW complexes were abolished when cell extracts were treated with the reducing agent  $\beta$ -mercaptoethanol, suggesting that they may involve disulfide bonds.

In contrast to Uba1, no HMW complexes involving Uba3Pk and Ubc12Pk were detected, either under homeostatic conditions or following H<sub>2</sub>O<sub>2</sub> treatment (**Fig. 3.4B, C**). Although no HMW forms of Uba3 or Ubc12 were detected, it was possible that these proteins could be oxidised at cysteine residues, but do not form intermolecular disulfides. The redox-sensitivity of Uba3 and Ubc12 was investigated further by using the cysteine alkylating agent AMS, which is able to modify reduced cysteines, but not cysteines involved in intramolecular disulfides, or that form sulfenic or sulfinic acid derivatives following oxidation. For every reduced cysteine, treatment with AMS adds ~0.5kDa to the M<sub>r</sub> of the protein. Uba3 includes 12 cysteine residues, meaning that AMS treatment adds ~6kDa to the M<sub>r</sub> of Uba3, if all cysteines are in the reduced form.



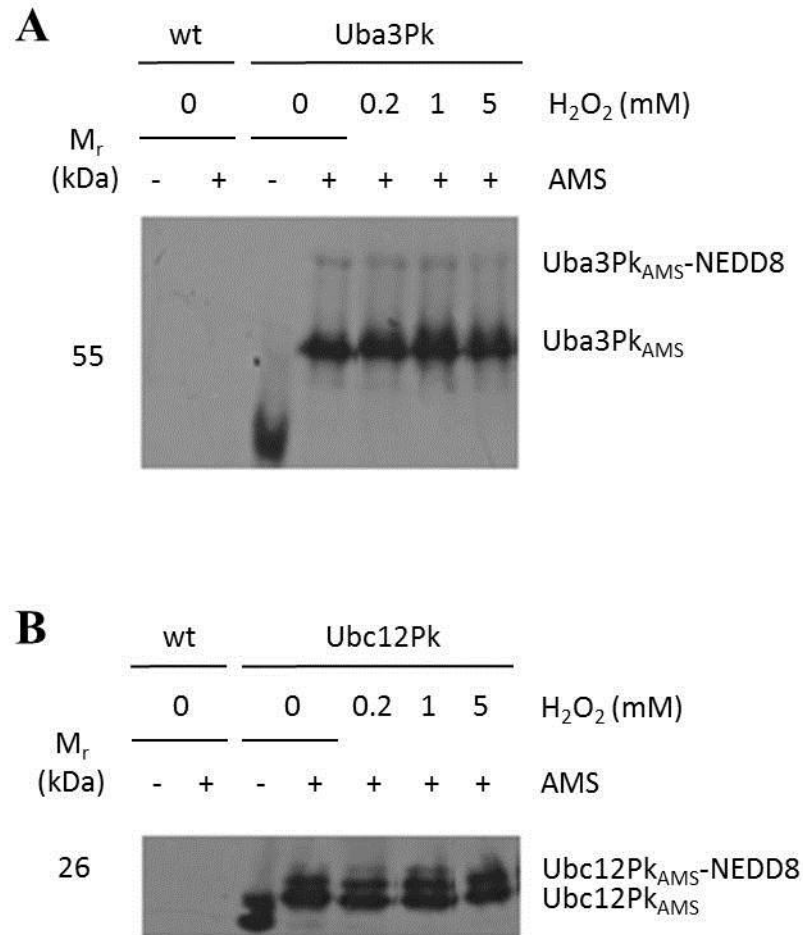
**Figure 3.4. Uba1, but not Uba3 or Ubc12, forms  $\beta$ -mercaptoethanol-sensitive HMW complexes in unstressed cells and following  $H_2O_2$  treatment.**

Cells expressing (A) Uba1Pk (MF32), (B) Uba3Pk (MF34), and (C) Ubc12Pk (MF36) were treated with the indicated concentrations of  $H_2O_2$  for 20 minutes, and cell extracts prepared by acid lysis. Extracts from Uba1Pk-expressing cells were prepared with or without the reducing agent  $\beta$ -mercaptoethanol, as indicated. Cell extracts were analysed by SDS-PAGE and western blotting under non-reducing conditions, using extracts from unstressed wild-type (wt; CHP428) cells as controls. Disulfide complexes involving Uba1Pk are highlighted by an asterisk. The positions on the blots including proteins of the  $M_r$  expected for E1-E2 complexes involving Uba3Pk and Ubc12Pk are indicated by red lines. All experiments were repeated, with the same results.

Ubc12 contains just two cysteine residues, resulting in an expected  $M_r$  increase of ~1kDa following AMS treatment. Importantly, if any cysteine residues were oxidised, the  $M_r$  increase would not be as great. To investigate Uba3 and Ubc12 oxidation, cells were exposed to a range of  $H_2O_2$  concentrations, and extracts prepared with or without AMS treatment. AMS-modified extracts were analysed by western blotting. When extracts from unstressed cells were treated with AMS, Ubc3Pk and Ubc12Pk showed a decrease in mobility through SDS-PAGE gels consistent with  $M_r$  increases of 6kDa and 1kDa, respectively (**Fig. 3.5**). When cells were exposed to  $H_2O_2$  prior to AMS treatment, there was no obvious change in these  $M_r$  shifts (**Fig. 3.5**), indicating that  $H_2O_2$  treatment does not alter the redox state of cysteines within Uba3 and Ubc12. It can be concluded that the cysteine residues of Uba3 and Ubc12 are resistant to oxidation in *S. pombe*.

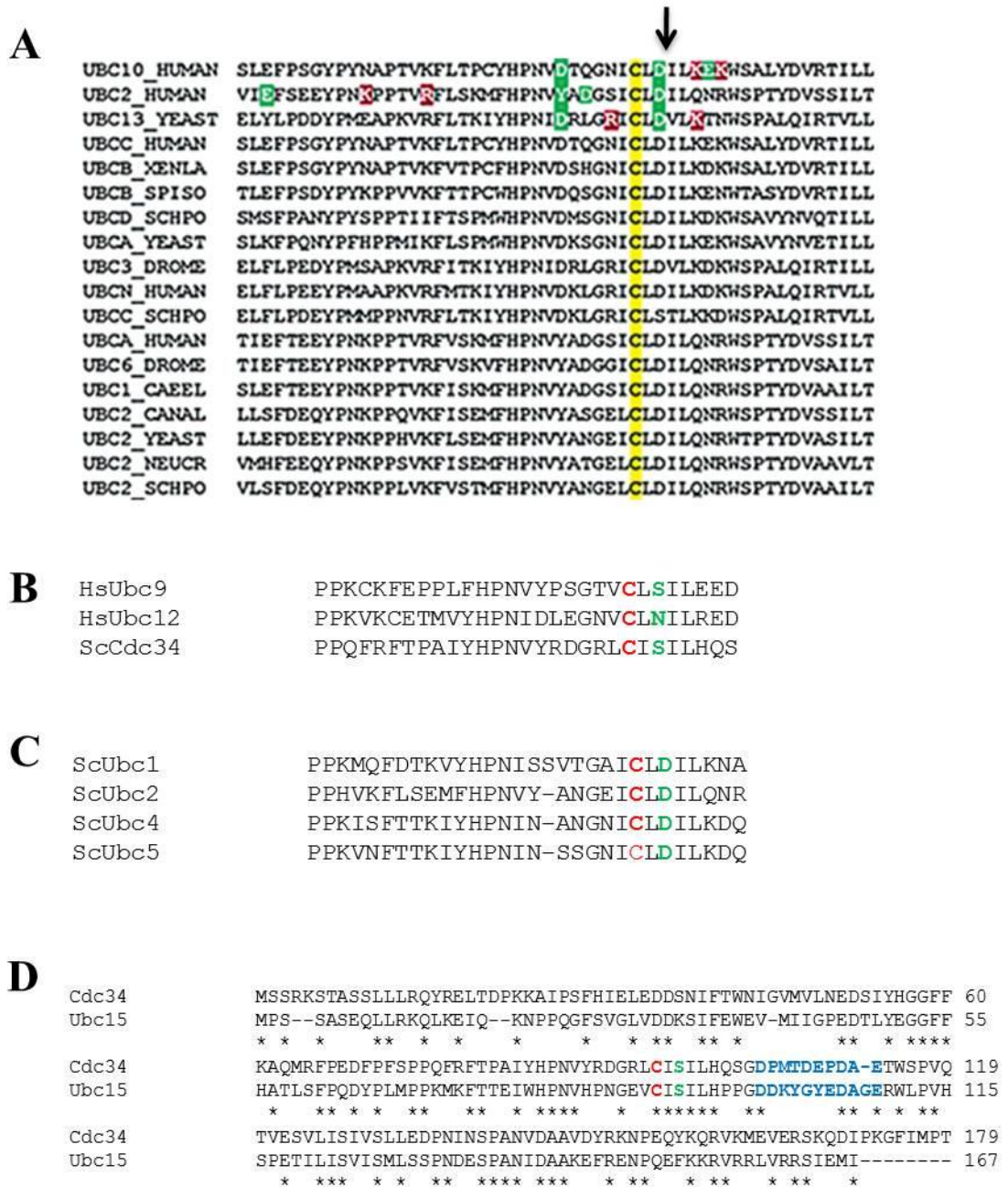
### **3.2.3. Identification of Ubc15 as a putative redox-regulated E2 in *S. pombe*.**

Here, Uba1 has been shown to form HMW complexes in fission yeast. This supports and extends our previous work describing the formation of Uba1-Cdc34 disulfide complexes during different oxidative stress conditions in budding yeast. Next, attempts were made to identify redox-sensitive E2s that may form disulfides with Uba1 in *S. pombe*. The catalytic cysteines of ubiquitin-specific E2s usually have high pKa values (Tolbert et al., 2005). This makes cysteine oxidation unlikely, as the formation of a thiolate anion at cytosolic pH requires a low pKa. The pKa of catalytic cysteines is strongly influenced by nearby amino acid residues. For example, positively-charged basic residues can stabilise thiolate anions, thus decreasing the relative pKa of the cysteine. In contrast, negatively-charged acidic residues decrease the likelihood of cysteine deprotonation. Significantly, a highly-conserved aspartate residue is located just downstream of the catalytic cysteine in many ubiquitin-specific E2s (**Fig. 3.6A**), and the structures of these E2s position the aspartate very close to the catalytic cysteine (Tolbert et al., 2005). However, this acidic residue is replaced by uncharged residues in redox-regulated E2s, such as mammalian Ubc9 and Ubc12, and budding yeast Cdc34 (**Fig. 3.6B**). In contrast to Cdc34, many other ubiquitin-specific E2s in *S. cerevisiae* have aspartate residues downstream of the catalytic cysteine (**Fig. 3.6C**), and are not redox-regulated (our unpublished data).



**Figure 3.5. Uba3 and Ubc12 are insensitive to oxidation at cysteine residues following H<sub>2</sub>O<sub>2</sub> treatment.**

(A) Uba3Pk (MF34) and (B) Ubc12Pk (MF36) cells were exposed to a range of H<sub>2</sub>O<sub>2</sub> concentrations for 20 minutes, and protein extracts were prepared by TCA lysis. Protein was also extracted from unstressed wild-type (wt; CHP428) cells, as a control. Protein extracts were prepared with or without the cysteine alkylating agent AMS, as indicated. AMS-modified proteins were analysed by non-reducing SDS-PAGE and western blotting, using anti-Pk antibodies. AMS-modified Uba3Pk (Uba3Pk<sub>AMS</sub>), Uba3Pk-NEDD8 (Uba3Pk<sub>AMS</sub>-NEDD8), Ubc12Pk (Ubc12Pk<sub>AMS</sub>) and Ubc12Pk-NEDD8 (Ubc12Pk<sub>AMS</sub>-NEDD8) are indicated above. Both experiments were repeated, with the same results.



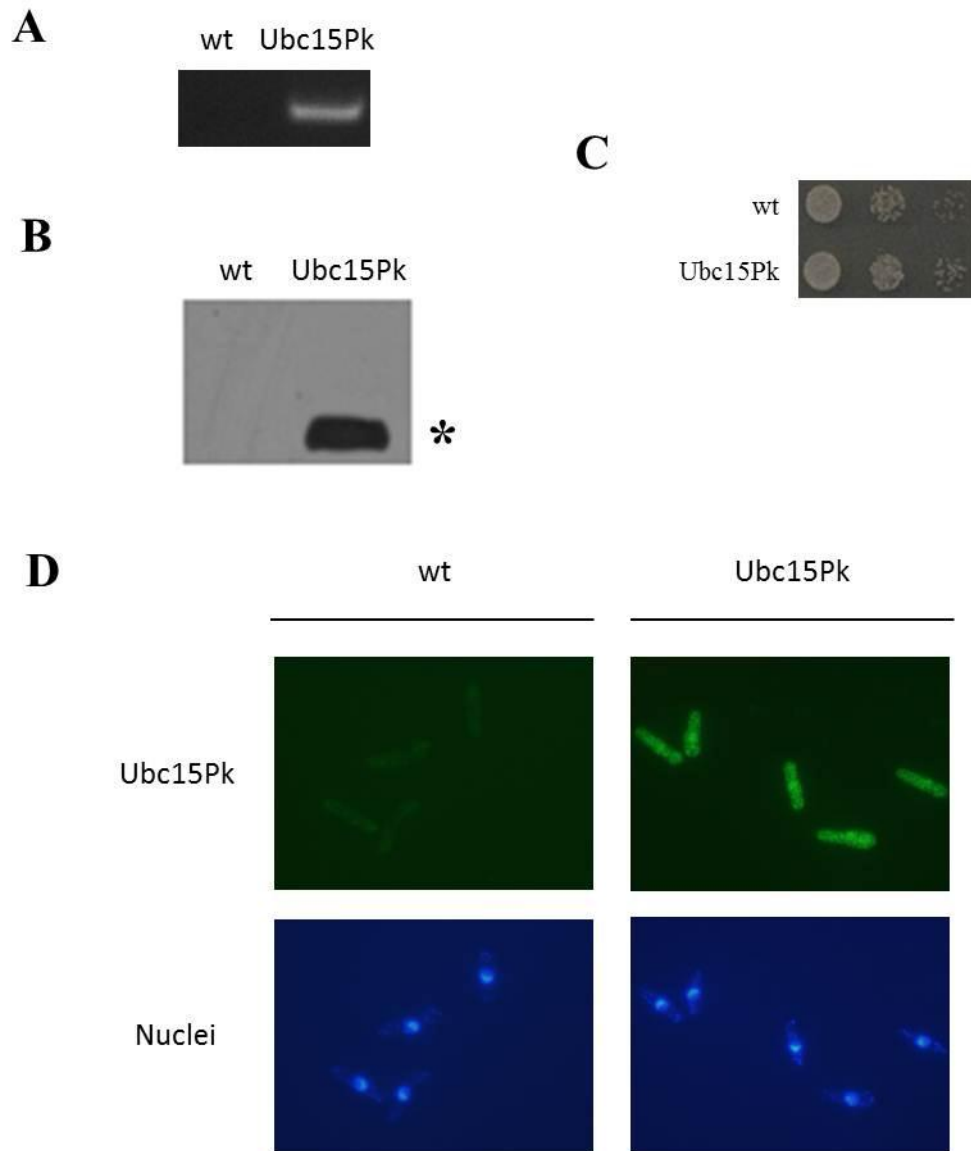
**Figure 3.6. Fission yeast Ubc15 is a possible redox-regulated orthologue of *S. cerevisiae* Cdc34.**  
**A.** The majority of eukaryotic E2s have catalytic cysteines with high pKa values, likely due to nearby acidic residues (adapted from Tolbert *et al.*, 2005). Catalytic cysteines are highlighted in yellow. A highly-conserved aspartate residue near the catalytic site is indicated by an arrow. **B.** Known redox-sensitive E2s lack the key aspartate residue located downstream of the catalytic cysteine. Amino acid sequence alignments of budding yeast Cdc34 (ScCdc34), human Ubc9 (HsUbc9) and human Ubc12 (HsUbc12) are shown. Catalytic cysteine residues are shown in red; key downstream residues at the position usually occupied by aspartate are shown in green. **C.** The majority of ubiquitin-specific E2s in *S. cerevisiae* include an aspartate residue downstream of the catalytic cysteine. Amino acid sequence alignments are shown above. Catalytic cysteine residues are shown in red; key downstream aspartate residues are shown in green. **D.** A putative redox-sensitive E2 was identified by BLAST search of the *S. pombe* proteome, using the amino acid sequence of budding yeast Cdc34. Ubc15 is the closest homologue of Cdc34, with 47% identity. Amino acid sequence alignments are shown above, and identical residues are indicated by asterisks. Catalytic cysteine residues are shown in red, conserved downstream serines are shown in green, and conserved acidic loops are shown in blue.

Cdc34 is an E2 which plays important roles in the control of the G1/S phase transition in *S. cerevisiae*. For example, Cdc34 facilitates the polyubiquitination and degradation of a Cdk, Sic1, during G1 phase, leading to the degradation of Sic1 and allowing the cell cycle to proceed (Glutzer et al., 1991; Goebel et al., 1988; King et al., 1996; Schwob et al., 1994). This mechanism appears to be conserved in mammalian cells (Pagano, 1997). However, no homologue of Cdc34 has been identified in *S. pombe*. Hence, to identify a potential Cdc34 homologue in *S. pombe*, a BLAST search of the *S. pombe* proteome was performed, using the amino acid sequence of Cdc34. This analysis identified a ubiquitin-specific E2, Ubc15, as a possible orthologue of Cdc34, with 47% identity (**Fig. 3.6D**). Although the downstream substrates of Ubc15 are currently unknown, Ubc15 was initially identified as an E2 which disrupts transcriptional silencing when overexpressed in *S. pombe* (Nielsen et al., 2002). This indicates that Ubc15 and Cdc34 may have different functions. However, the possibility that Ubc15 is a Cdc34 orthologue is supported by the presence of an acidic loop located downstream of the catalytic cysteine of Ubc15 (**Fig. 3.6D**), which is conserved in Cdc34 orthologues and is important for polyubiquitin chain formation (Petroski and Deshaies, 2005). Although Ubc15 lacks the acidic C-terminal ‘tail’ of Cdc34, the region immediately downstream of the catalytic cysteines is exactly the same, with a serine residue at the position occupied by an aspartate in other E2s. Although there are likely to be other structural factors contributing to the redox-sensitivity of catalytic cysteine residues, these findings led us to investigate the possibility that Ubc15 forms HMW disulfide complexes in *S. pombe*.

#### **3.2.4. Ubc15 forms a $\beta$ -mercaptoethanol-sensitive HMW complex during severe oxidative stress.**

To investigate the redox-sensitivity of Ubc15, the protein was tagged at the C-terminus with three copies of the Pk epitope and expressed from the chromosomal locus, using the strategy described in **Fig. 3.2**. First, the integration tagging plasmid pRIP42PkC-*ubc15* was constructed (see Section 2.1.5.1), linearised and introduced into CHP429 wild-type cells to allow integration into the *ubc15*<sup>+</sup> gene locus. Integration of pRIP42PkC-*ubc15* into the *ubc15*<sup>+</sup> locus was confirmed by PCR (**Fig. 3.7A**), and the expression of Ubc15Pk was confirmed by western blotting (**Fig. 3.7B**). Importantly, Pk-





**Figure 3.7. Expressing Pk epitope-tagged Ubc15 has no effect on cell viability, and indirect immunofluorescence reveals that Ubc15 is localised throughout the cell, but is particularly abundant in the nucleus.**

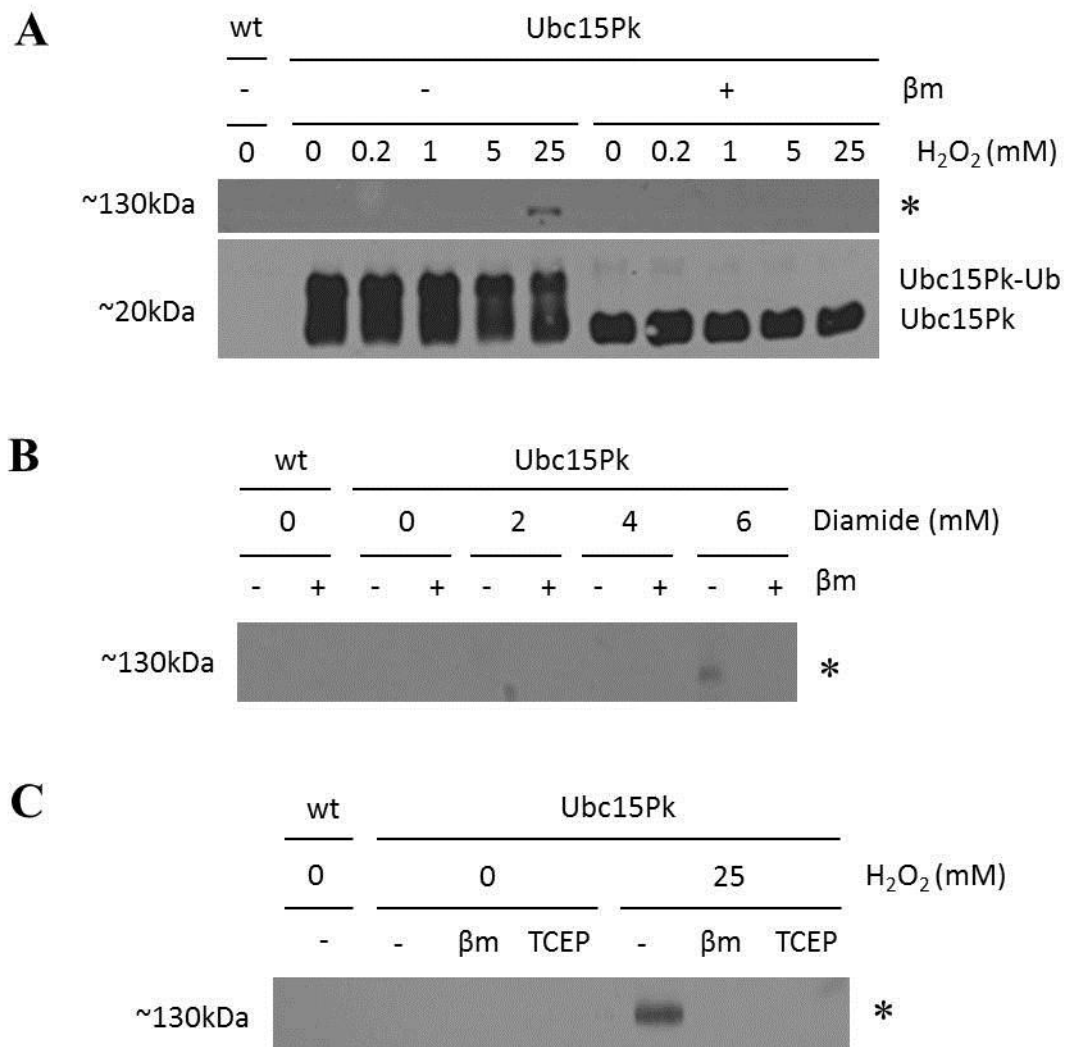
**A.** To confirm integration of pRIP42PkC, gDNA was extracted from wild-type (wt; CHP429) cells and from cells transformed with linearised, recombinant pRIP42PkC to tag Ubc15 (MF58). Integration of pRIP42PkC at the *ubc15*<sup>+</sup> locus was confirmed by PCR using the forward primer Ubc15chkf, which binds to a site within the ORF, and the reverse primer nmtend, which binds to a site within the nmt sequence of pRIP42PkC (see Table 2.2), and gDNA extracts as templates. PCR products were analysed by agarose gel electrophoresis, and a ~600bp product confirmed integration of pRIP42PkC. **B.** Protein extracts from wild-type (wt; CHP429) cells and a strain expressing Ubc15Pk (MF58) were prepared and analysed by western blotting. Pk epitope-tagged Ubc15 (~20kDa) is indicated by an asterisk. **C.** Ten-fold serial dilutions of mid-log phase wild-type (wt; CHP428) and Ubc15Pk (MF58) cells were spotted onto solid YE5S media. Plates were incubated at 30°C for three days. **D.** To investigate the cellular localisation of Ubc15, mid-log phase wild-type (wt; CHP428) and Ubc15Pk (MF58) cells were fixed with 3.7% paraformaldehyde. Ubc15 was visualised by indirect immunofluorescence using mouse anti-Pk antibodies and Alexa Fluor®-conjugated anti-mouse antibodies. Nuclei were visualised by DAPI staining.

tagging of Ubc15 caused no obvious effects on cell viability (**Fig. 3.7C**). The majority of Cdc34 protein is found in the nucleus in *S. cerevisiae* (Mathias et al., 1998). If Ubc15 is an orthologue of Cdc34, Ubc15 would also be expected to localise to the nucleus. The localisation of Ubc15Pk was therefore investigated by indirect immunofluorescence (**Fig. 3.7D**). Although Ubc15 was present in the cytoplasm, stronger signals were detected in the nucleus. This supports the possibility that Ubc15 is a Cdc34 orthologue, and further supports previous work implicating Ubc15 in the regulation of transcriptional silencing in fission yeast (Nielsen et al., 2002), which would likely require Ubc15 to localise to the nucleus.

To investigate the possibility that Ubc15 may be more redox-sensitive than other E2s, and may therefore form HMW disulfide complexes following oxidation, Ubc15Pk cells were treated with different H<sub>2</sub>O<sub>2</sub> concentrations, and cellular extracts prepared and analysed by western blotting. In contrast to Uba3Pk and Ubc12Pk, treatment of Ubc15Pk cells with 25mM H<sub>2</sub>O<sub>2</sub> resulted in the formation of a HMW species of ~130kDa (**Fig. 3.8A**). Significantly, this is the approximate M<sub>r</sub> that would be expected for a complex involving Uba1 and Ubc15. Furthermore, treatment of cellular extracts with  $\beta$ -mercaptoethanol abolished complex formation, suggesting that the HMW species involves disulfide bonds (**Fig. 3.8A**). During our investigations in *S. cerevisiae*, treatment of cells with the GSH-depleting agent diamide also caused the formation of Uba1-Cdc34 disulfide complexes (our unpublished data). Similarly, high concentrations of diamide triggered the formation of  $\beta$ -mercaptoethanol-sensitive HMW complexes involving Ubc15Pk (**Fig 3.8B**). In addition, HMW complex formation was abolished by treatment of cellular extracts with TCEP, a disulfide-specific reducing agent (**Fig. 3.8C**), further supporting the conclusion that these HMW complexes form due to cysteine oxidation and disulfide formation. Importantly, Ubc15 only includes a single cysteine residue, suggesting that the formation of any HMW disulfide complexes would require oxidation of the catalytic cysteine.

Based on our studies in *S. cerevisiae*, it might be expected that the ~130kDa complex involving Ubc15Pk also includes Uba1. In support of this, Uba1 is also present in ~130kDa complexes (**Fig. 3.4A**). However, Uba1-Ubc15 disulfide complex formation has not been shown conclusively. If Ubc15Pk did not form a HMW complex following H<sub>2</sub>O<sub>2</sub> treatment in a Ubc15Pk  $\Delta uba1$  strain, it would provide strong evidence that the complex involves both Uba1 and Ubc15. However, *uba1*<sup>+</sup> is an essential gene,





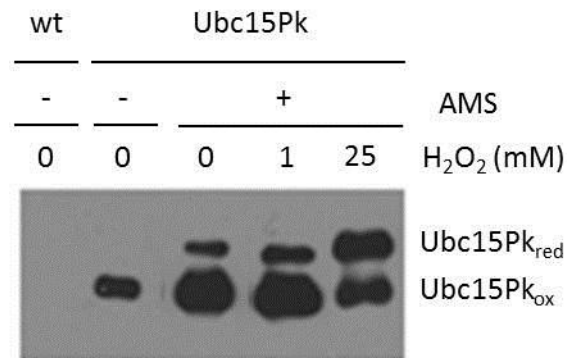
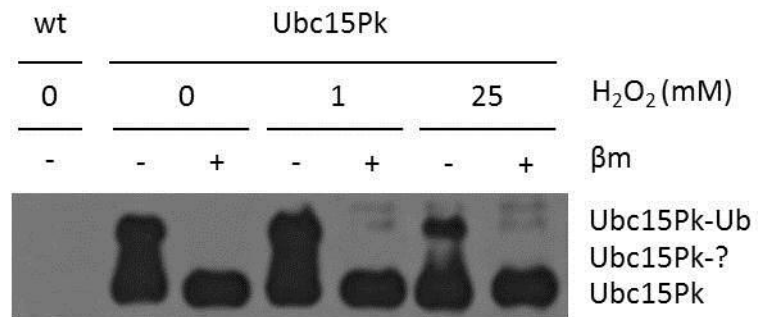
**Figure 3.8. Ubc15 forms HMW disulfide complexes at high levels of oxidative stress.**

**A, B.** Ubc15Pk (MF58) and wild-type (wt; CHP429) cells were treated with the indicated concentrations of **(A)**  $H_2O_2$  and **(B)** diamide for 20 minutes. Cell extracts were prepared by TCA lysis in the presence and absence of the reducing agent  $\beta$ -mercaptoethanol, as indicated, and analysed by western blotting, using anti-Pk antibodies. Free and ubiquitin-bound forms of Ubc15Pk are indicated. HMW complexes are indicated by asterisks. These results are representative of three different experiments. **C.** Ubc15Pk (MF58) and wild-type (wt; CHP429) cells were treated with the indicated concentrations of  $H_2O_2$  for 20 minutes. Cell extracts were prepared by TCA lysis in the presence and absence of  $\beta$ -mercaptoethanol or the disulfide-specific reducing agent TCEP, as indicated, and analysed by western blotting using anti-Pk antibodies. Ubc15 disulfide complexes are indicated by an asterisk. This result is representative of three different experiments.

preventing the construction of a *Δuba1* strain. An alternative approach would be to express an epitope-tagged Uba1 protein in Ubc15Pk cells, and investigate whether this results in a  $M_r$  shift in HMW complexes formed during  $H_2O_2$  treatment. Decreased mobility of HMW complexes would provide strong evidence for the involvement of Uba1. Nevertheless, the results presented here support the hypothesis that Cdc34-like proteins are redox-sensitive in evolutionarily divergent organisms, and may form HMW complexes with Uba1.

### 3.2.5. Ubc15 may be glutathionylated during mild oxidative stress.

It has now been established that Ubc15 forms HMW complexes during severe oxidative stress, and that such complexes are likely to involve a disulfide bond. However, the concentrations of  $H_2O_2$  required for Ubc15 complex formation were much higher than the concentrations required for Cdc34 complex formation in *S. cerevisiae* (our unpublished data). This suggests that the catalytic cysteine of Ubc15 is far less sensitive to oxidation than the catalytic cysteine of Cdc34. To investigate this possibility, the oxidation state of the Ubc15 catalytic cysteine residue was analysed at different concentrations of  $H_2O_2$  using the alkylating agent AMS (see Section 3.2.2). Cells expressing Ubc15Pk were treated with different concentrations of  $H_2O_2$ , and cellular extracts prepared and treated with AMS. Unexpectedly, only a small pool of Ubc15 is modified by AMS under homeostatic conditions or during mild oxidative stress (**Fig. 3.9A**). This suggests that levels of apparently-oxidised Ubc15 (Ubc15<sub>ox</sub>) are greater than levels of reduced Ubc15 (Ubc15<sub>red</sub>). This can be explained, at least in part, by roughly half of cellular Ubc15 forming thioesters (Ubc15-Ub) under normal conditions (**Fig. 3.8A**). This would effectively ‘shield’ the catalytic cysteine from modification by AMS. However, far more Ubc15<sub>ox</sub> than Ubc15<sub>red</sub> was detected under homeostatic conditions, implying that another significant pool of Ubc15 has its catalytic cysteine residue shielded from AMS modification. In contrast, far more Ubc15 protein is modified with AMS following exposure to 25mM  $H_2O_2$  (**Fig. 3.9A**). This is consistent with a loss of cysteine modification at 25mM  $H_2O_2$ , thus increasing AMS-modification of Ubc15 during severe stress.

**A****B**

**Figure 3.9. Ubc15 may be glutathionylated to prevent HMW complex formation during mild oxidative stress.**

**A.** Ubc15Pk (MF58) cells were treated with a range of concentrations of H<sub>2</sub>O<sub>2</sub> for 1 minute, and protein extracts prepared by TCA lysis. Extracts from wild-type (wt; CHP429) cells expressing untagged protein were also prepared. Protein extracts were treated with the cysteine-modifying agent AMS, where indicated. The oxidation state of the Ubc15 catalytic cysteine was then investigated by SDS-PAGE and western blotting under reducing conditions, using anti-Pk antibodies. AMS-modified Ubc15Pk (Ubc15Pk<sub>red</sub>) and unmodified Ubc15Pk (Ubc15Pk<sub>ox</sub>) are indicated above. This experiment was repeated and gave the same result. **B.** Ubc15Pk (MF58) cells were treated with the indicated concentrations of H<sub>2</sub>O<sub>2</sub> for 20 minutes. Cell extracts were prepared in the presence and absence of the reducing agent  $\beta$ -mercaptoethanol, as indicated. Extracts from wild-type (wt; CHP429) cells expressing untagged protein were also prepared. These extracts were analysed by western blotting, using anti-Pk antibodies. Free Ubc15Pk, ubiquitin-loaded Ubc15Pk (Ubc15Pk-Ub) and an unknown modification of Ubc15Pk (Ubc15Pk-?) are indicated. This experiment was repeated and gave the same result.

It is possible that the catalytic cysteine of Ubc15 is glutathionylated under normal conditions, and that this glutathionylation is lost when cells are exposed to high concentrations of  $\text{H}_2\text{O}_2$ . This could be due to the increased oxidation of other cellular proteins at high concentrations of  $\text{H}_2\text{O}_2$ , leading to glutathionylation of these proteins and thus decreasing the level of glutathione available to modify the catalytic cysteine of Ubc15. It has been shown that ubiquitin-specific E2s are modified by glutathionylation to protect cysteines from irreversible oxidation, as glutathionylation can be quickly reversed by a second GSH molecule (Jahngen-Hodge et al., 1997). Moreover, western blotting experiments suggest that a modified form of Ubc15 with a lower  $M_r$  than ubiquitin-loaded Ubc15 is lost during severe oxidative stress (**Fig. 3.8A**). To investigate this possibility further, cells expressing Ubc15Pk were again treated with different concentrations of  $\text{H}_2\text{O}_2$ , and cell extracts were analysed by western blotting. Again, exposure to high concentrations of  $\text{H}_2\text{O}_2$  resulted in the disappearance of a  $\beta$ -mercaptoethanol-sensitive modified form of Ubc15, as well as a decrease in the level of ubiquitin-loaded Ubc15 (**Fig. 3.9B**).

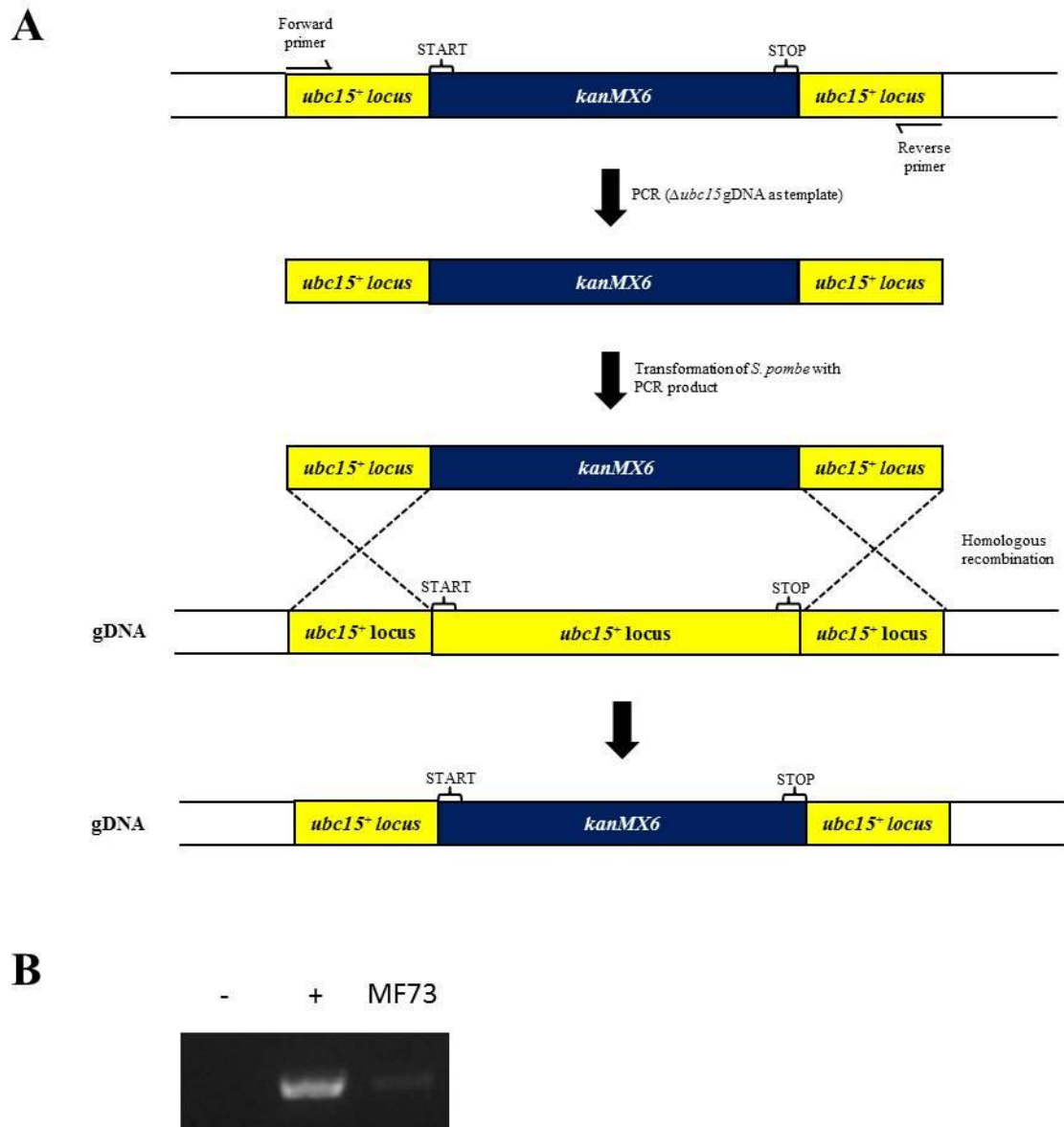
The  $M_r$  of the modified form of Ubc15 is consistent with glutathionylation. At low concentrations of  $\text{H}_2\text{O}_2$ , glutathionylation of the catalytic cysteine residue of Ubc15 may protect against irreversible hyperoxidation, as well as preventing HMW disulfide complex formation. However, it is important to note that glutathionylation of the catalytic cysteine would inhibit Ubc15 function in the same way as HMW disulfide complex formation, as shown for other ubiquitin/Ubl conjugation pathways (Bossis and Melchior, 2006; Kumar et al., 2007). If a downstream substrate of Ubc15 were identified, it would be interesting to investigate how different concentrations of  $\text{H}_2\text{O}_2$  affect the ubiquitination of this substrate, and therefore compare the extent to which Ubc15 is inhibited by glutathionylation or HMW disulfide complex formation. Interestingly, high concentrations of  $\text{H}_2\text{O}_2$  also appear to decrease the levels of ubiquitin-loaded Ubc15 (**Fig. 3.8A and 3.9B**), which may result in decreased ubiquitination of downstream substrates at high  $\text{H}_2\text{O}_2$  concentrations.

### **3.3. Ubc15 functions in oxidative stress responses.**

### 3.3.1. Ubc15 is required for resistance to diamide.

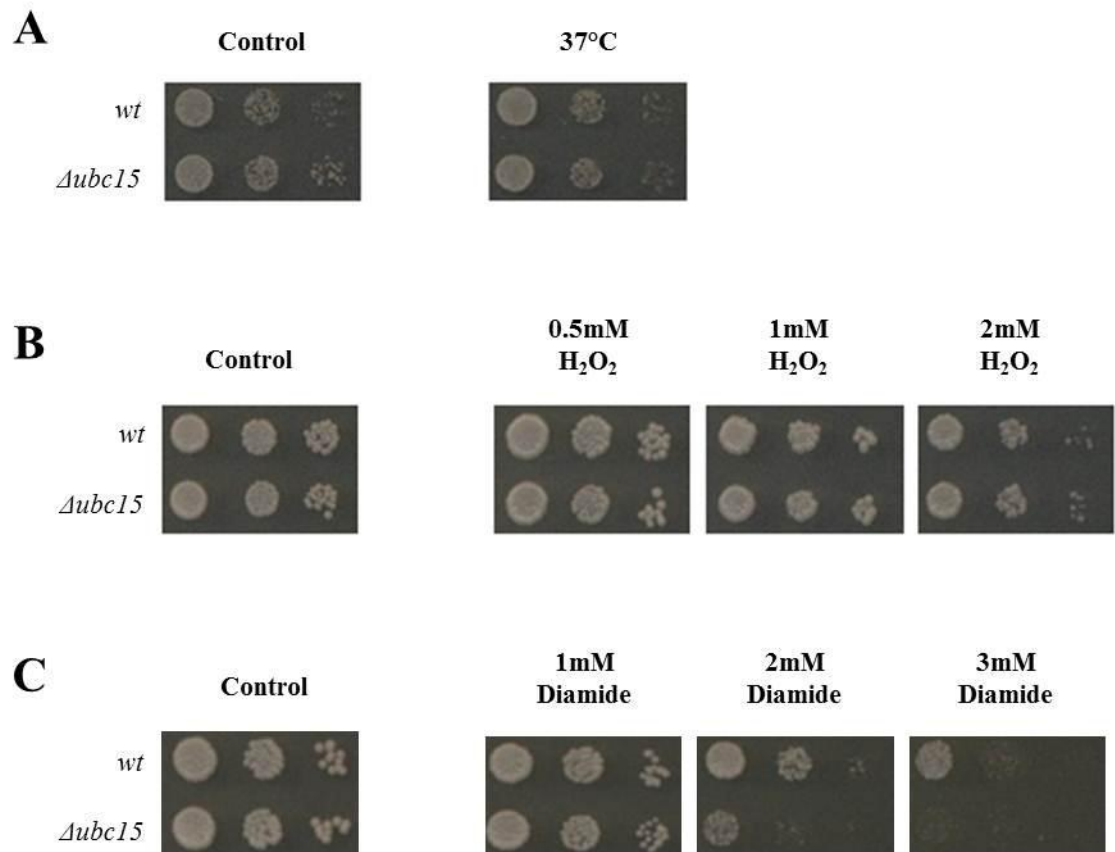
Having established that Ubc15 is redox-sensitive, the potential role of Ubc15 in oxidative stress responses was investigated. In contrast to budding yeast Cdc34, the downstream substrates of Ubc15 are unknown. Consequently, it is currently difficult to investigate the effect of oxidative stress on substrate ubiquitination by Ubc15, and the resulting effects on cellular processes. However, while *CDC34* is essential for cell cycle progression in *S. cerevisiae* (Byers and Goetsch, 1974), *ubc15*<sup>+</sup> is not essential in *S. pombe* (Nielsen et al., 2002). Furthermore, a Bioneer deletion library includes a  $\Delta$ *ubc15* strain (BG\_2361) with a *KanMX6* cassette at the *ubc15*<sup>+</sup> (*SPBC1105.09*) locus. Hence, this  $\Delta$ *ubc15* strain could be used to investigate the potential role of Ubc15 in responses to oxidative stress. However, no wild-type background strain was available, preventing the use of this strain to investigate how loss of Ubc15 affects stress resistance. Consequently, the strategy outlined in **Fig. 3.10A** was used to construct a  $\Delta$ *ubc15* strain using our own strain backgrounds. A  $\Delta$ *ubc15* cassette was generated by PCR and introduced into CHP429 wild-type cells. This cassette integrated into the genome via homologous recombination, thus replacing the *ubc15*<sup>+</sup> ORF with a *KanMX6* cassette and conferring resistance to G-418. Incorporation of *KanMX6* at the correct locus was confirmed by PCR (**Fig. 3.10B**). Having successfully generated a  $\Delta$ *ubc15* mutant strain in a CHP429 background, the growth of  $\Delta$ *ubc15* cells on solid YE5S media was investigated. As expected,  $\Delta$ *ubc15* cells displayed similar growth to wild-type cells at 30°C, and there was no evidence of slower growth at 37°C, relative to the wild-type control (**Fig. 3.11A**). Next, the sensitivity of  $\Delta$ *ubc15* cells to a range of oxidative stress agents was examined. Interestingly,  $\Delta$ *ubc15* cells did not display increased sensitivity to H<sub>2</sub>O<sub>2</sub> (**Fig. 3.11B**), but  $\Delta$ *ubc15* cells were more sensitive to diamide than the wild-type control (**Fig. 3.11C**).

The above results demonstrate that  $\Delta$ *ubc15* cells are more sensitive to diamide than wild-type cells. It is unclear how this relates to the formation of HMW disulfide complexes involving Ubc15. It is possible that the formation of these disulfide complexes is important for short-term resistance to severe oxidative stress, as the concentrations of diamide (or H<sub>2</sub>O<sub>2</sub>) required to trigger complex formation are higher than concentrations preventing the long-term growth of wild-type cells (compare **Fig.**



**Figure 3.10. Strategy for the construction of a  $\Delta ubc15$  strain.**

**A.** To construct a  $\Delta ubc15$  strain, gDNA was extracted from a Bioneer  $\Delta ubc15$  strain (BG\_2361), and used as a template for PCR. Oligonucleotide primers binding ~250bp upstream of the *ubc15<sup>+</sup>* START codon (Ubc15delf) and ~250bp downstream of the *ubc15<sup>+</sup>* STOP codon (Ubc15delr) were used to amplify the *KanMX6* cassette, flanked by regions of homology to the *ubc15<sup>+</sup>* locus. The PCR product was introduced into wild-type (CHP428) cells, and integrated into the genome by homologous recombination. Positive transformants were selected by growth on YE5S plates containing G-418. **B.** To confirm  $\Delta ubc15$  strain construction, gDNA was prepared from a G-418-resistant colony (MF73), and from wild-type (CHP428; -) and Bioneer  $\Delta ubc15$  (BG\_2361; +) cells. PCR was performed using these DNA extracts as templates, using the forward primer Ubc15delf and the reverse primer KanMX6delchk, which binds to a site within *KanMX6* (see Table 2.2). PCR products were analysed by agarose gel electrophoresis, and a product of ~800bp was taken as confirmation of strain construction.



**Figure 3.11. *Δubc15* display increased sensitivity to diamide, but not to H<sub>2</sub>O<sub>2</sub>.**

To examine the growth of *Δubc15* cells, ten-fold serial dilutions of mid-log phase growing wild-type (*wt*; CHP429) and *Δubc15* (MF73) cells were spotted onto (A) solid YE5S media and incubated at 30°C or 37°C for three days. To investigate whether Ubc15 is required for oxidative stress resistance, cells were spotted onto solid media containing the indicated concentrations of (B) H<sub>2</sub>O<sub>2</sub> or (C) diamide and incubated at 30°C for three days. These results are representative of three experiments.

**3.8B** and **Fig. 3.11B**). To gain further insight into the role of Ubc15 in oxidative stress responses, it will be important to identify downstream substrates of Ubc15, and analyse how their ubiquitination is affected by different levels of stress.

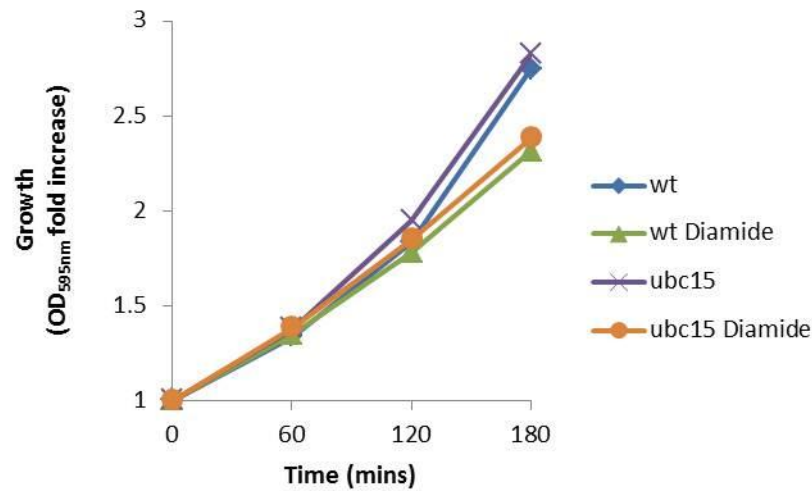
### **3.3.2. Ubc15 does not control cell growth during short-term exposure to diamide.**

Although *Δubc15* cells displayed increased sensitivity to 2mM diamide than wild-type cells when grown on solid YE5S media (**Fig. 3.11C**), these sensitivity tests do not show whether *Δubc15* cells have a slower growth rate or a decreased survival rate when they are exposed to diamide. Hence, to investigate whether Ubc15 is important for the control of cell growth during short-term exposure to diamide, *Δubc15* and wild-type cells growing in liquid media were treated with 2mM diamide, and growth rates were examined by monitoring OD<sub>595nm</sub> of the cultures. These cultures had similar growth rates, and diamide treatment decreased the growth rate of both cultures equally (**Fig. 3.12**). Additionally, the survival rates of wild-type and *Δubc15* cells were investigated (see Section 2.2.2). Treatment with 2mM diamide did not decrease the survival rates of either wild-type or *Δubc15* cells over the first three hours of exposure, and prolonged diamide exposure decreased the survival rate of both strains equally (data not shown). Taken together, these data indicate that loss of Ubc15 does not have immediate effects on cell growth or survival during exposure to diamide. Thus, although previous sensitivity tests showed that *Δubc15* cells are more sensitive to diamide than wild-type cells (**Fig. 3.11B**), it is unclear whether loss of Ubc15 affects the growth or survival of *S. pombe* cells following diamide exposure. It is possible that *Δubc15* cells are no more sensitive to short periods of diamide exposure than wild-type cells, and that prolonged exposure is required for differences in sensitivity to be detected.

### **3.3.3. Ubc15 does not control Pap1 levels.**

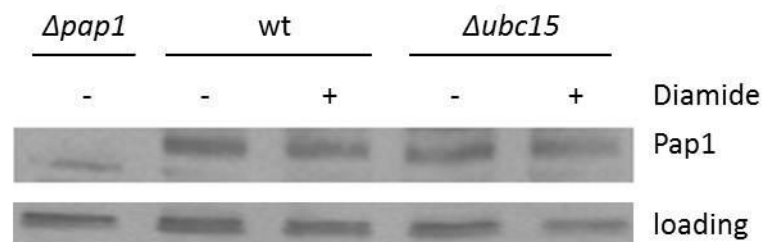
The transcription factor Pap1 is activated by many oxidative stress agents, and regulates the expression of numerous genes involved in oxidative stress responses in *S. pombe*





**Figure 3.12. Loss of Ubc15 does not affect cell growth during short-term exposure to diamide.**

To investigate the effect of diamide treatment on the growth of  $\Delta ubc15$  (MF73) and wild-type (wt; CHP429) cells, mid-log phase cultures were treated with 2mM diamide, or untreated, and incubated at 30°C. Growth was monitored by measuring the OD<sub>595nm</sub> of cultures at one-hour intervals following the addition of diamide. Fold growth was calculated relative to the OD<sub>595nm</sub> value at Time 0 for each culture. The above result is representative of three independent experiments.



**Figure 3.13. Ubc15 does not influence Pap1 protein levels.**

To investigate the effect of  $\Delta ubc15$  on Pap1 protein levels, mid-log phase wild-type (wt; CHP429) and  $\Delta ubc15$  (MF73) cells were treated with 2mM diamide for 20 minutes, before preparing cellular extracts. Extracts were also prepared from unstressed cells and from  $\Delta pap1$  (JP178) cells. To examine Pap1 protein levels, cell extracts were examined by SDS-PAGE and western blotting using polyclonal anti-Pap1 antibodies (Quinn *et al.*, 2002). A non-specific band was used as a loading control.

(see Section 1.3.1). Ubr1 has recently been identified as an E3 ligase that promotes the degradation of active, nuclear Pap1 (Kitamura et al., 2011). However, the upstream E2 has not been identified, raising the possibility that Ubc15 may play a role in the degradation of Pap1. In the absence of the upstream E2 or E3, Pap1 would become activated during oxidative stress as in wild-type cells, but active Pap1 may not be degraded, and could remain in the nucleus even after ROS homeostasis has been restored. This would result in aberrant expression of stress response genes, possibly leading to decreased viability. To investigate the possibility that Ubc15 is involved in the degradation of active Pap1, wild-type and *Δubc15* cells were treated with diamide. Cell extracts were prepared and Pap1 protein levels were examined by western blotting. Significantly, loss of Ubc15 does not appear to affect Pap1 protein levels, either under homeostatic conditions or following diamide treatment (**Fig. 3.13**). Taken together, these data show that Ubc15 influences the cellular response to diamide, but not through the control of Pap1 levels.

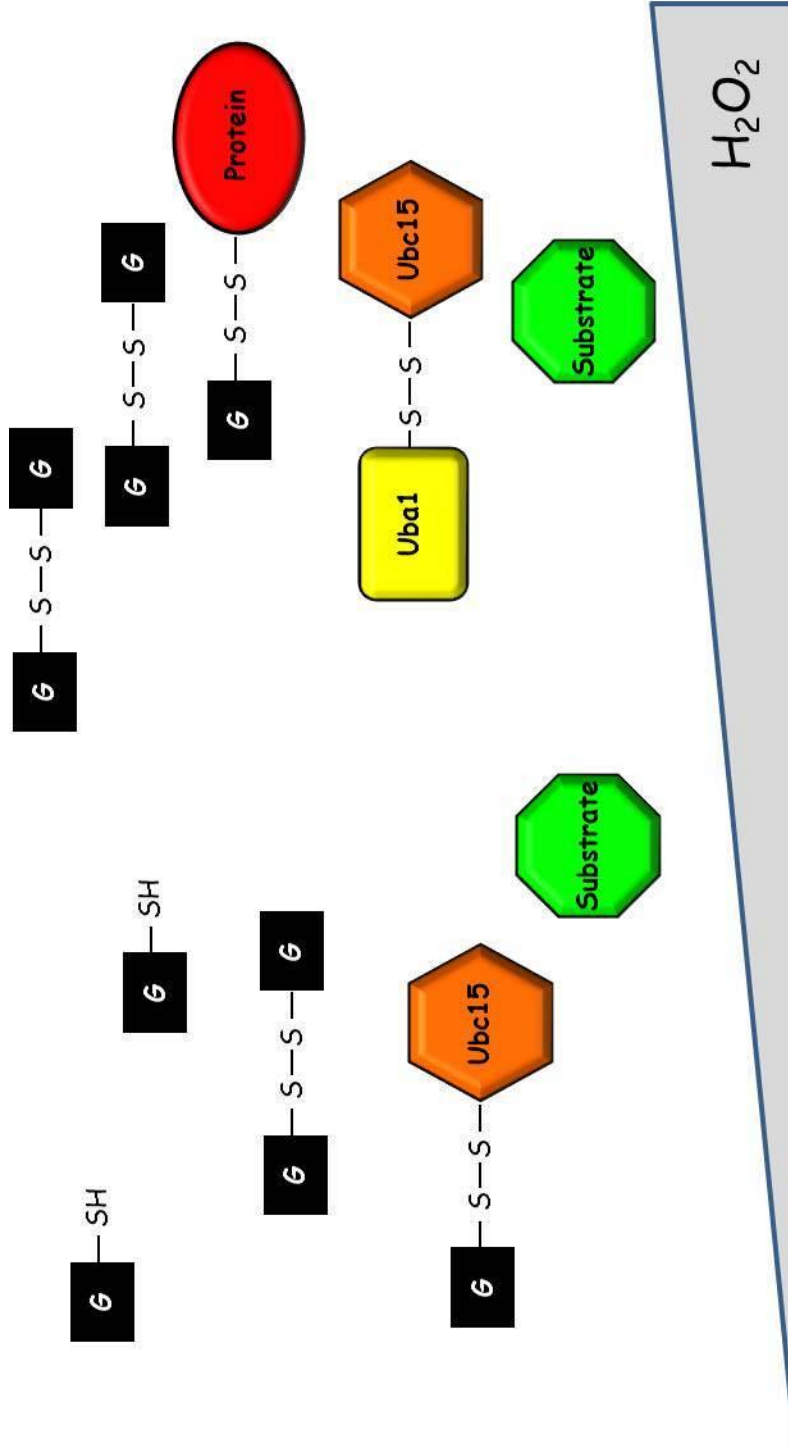
### 3.4. Discussion

In this chapter, it has been shown for the first time that specific enzymes functioning in ubiquitination can be redox-regulated during oxidative stress in fission yeast. Together with published work showing redox regulation of Ubl conjugation in mammalian cells, and our own data revealing redox regulation of ubiquitination in budding yeast, these results demonstrate that specific E1s and E2s are sensitive to oxidation in evolutionarily diverse eukaryotes.

In *S. cerevisiae*, Cdc34 is oxidised by low concentrations of H<sub>2</sub>O<sub>2</sub>, leading to stabilisation of Sic1 and a cell cycle delay in G1 phase (our unpublished data). This is likely to be important for oxidative stress resistance, as delaying the onset of S phase could allow the cell to restore redox homeostasis and prevent oxidative DNA damage just prior to DNA replication. In mammalian cells, the inhibition of SUMOylation by H<sub>2</sub>O<sub>2</sub> could promote the activity of antioxidant transcription factors, thus allowing cells

to mount a transcriptional response to oxidative stress (Bossis and Melchior, 2006). Thus, redox regulation of ubiquitin/Ubl conjugation could be important for resistance to oxidative stress in eukaryotes. The ubiquitin-specific E2 Ubc15 forms inhibitory disulfide complexes when cells are exposed to oxidative stress agents. The  $M_r$  of these complexes suggests that they also involve Uba1. However, the majority of Uba1 does not become sequestered in HMW complexes during oxidative stress, which could allow Uba1 to continue to transfer ubiquitin to other E2s. Furthermore, although cells must be treated with high concentrations of stress agents before Ubc15 complexes can be detected, Ubc15 may be glutathionylated at lower levels of stress, which could also inhibit its activity. Indeed, oxidative stress has been shown to increase the glutathionylation of E1s and E2s in mammalian cells, possibly to prevent irreversible oxidative modifications of catalytic cysteine residues (Jahngen-Hodge et al., 1997). Increasing levels of oxidative stress may increase the glutathionylation of other cellular proteins, thus decreasing the glutathione available to modify Ubc15 and favouring the formation of intermolecular disulfides instead of glutathionylation. This model is represented in **Fig. 3.14**.

Critically, the downstream substrates of Ubc15 are unknown, making it difficult to examine the effects of oxidative stress on Ubc15-dependent ubiquitination. This also makes it difficult to investigate how redox regulation of Ubc15 affects oxidative stress response mechanisms in *S. pombe*. Given that Ubc15 is an orthologue of Cdc34, and that Cdc34 facilitates the ubiquitination and degradation of Sic1, it is possible that the fission yeast Sic1 orthologue, Rum1, is targeted for degradation by Ubc15. Rum1 inhibits Cdc2, the sole Cdk in *S. pombe* (Labib and Moreno, 1996; Sanchez-Diaz et al., 1998). Cdc2 forms complexes with B-type cyclins throughout the cell cycle. Due to this, Rum1 might be expected to halt the cell cycle at many different stages. However, Rum1 levels only become sufficient to inhibit Cdc2 activity during G1 phase (Labib and Moreno, 1996). Indeed, one of the key functions of Rum1 is to prevent S phase entry until cells have reached a certain mass (Labib and Moreno, 1996). Rum1 levels are largely regulated by protein stability, and the ubiquitin-proteasome system maintains low Rum1 levels from the G1-S phase boundary until the end of M phase (Benito et al., 1998). If Ubc15 is the E2 facilitating ubiquitination of Rum1, inhibition of Ubc15 could cause Rum1 accumulation, resulting in a transient cell cycle arrest to allow cells to recover from oxidative stress, as in *S. cerevisiae*. However, *Δubc15* cells show no defects in cell cycle progression, suggesting that this is unlikely. Moreover, *Δubc15*



**Figure 3.14. A model for the redox regulation of Ubc15 at different levels of  $H_2O_2$ .** At low concentrations of  $H_2O_2$ , the catalytic cysteine residue of Ubc15 is oxidised and glutathionylated. This modification may protect the catalytic cysteine from hyperoxidation, allowing reactivation of the E2. At higher concentrations of  $H_2O_2$ , many other proteins may become oxidised and glutathionylated. This could sequester much of the cellular supply of glutathione. Thus, oxidation of the catalytic cysteine of Ubc15 may cause the E2 to form inhibitory disulfides with specific interacting proteins, such as Uba1.

cells do not display increased sensitivity to H<sub>2</sub>O<sub>2</sub>, implying that Ubc15 is not involved in the regulation of responses to H<sub>2</sub>O<sub>2</sub>. Alternatively, it is possible that Ubc15 is important for survival following short-term exposure to high concentrations of H<sub>2</sub>O<sub>2</sub>, but not long-term exposure to lower concentrations of H<sub>2</sub>O<sub>2</sub>. Indeed, *S. pombe* cells can survive short-term exposure to H<sub>2</sub>O<sub>2</sub> concentrations as high as 25mM (Day et al., 2012), and it is possible that redox regulation of Ubc15 is important for this survival. Additionally, *Δubc15* cells display increased sensitivity to diamide, suggesting that Ubc15 may regulate GSH levels. Clearly, these possibilities require further investigation.

Previous investigations have shown that enzymes facilitating SUMOylation and neddylation can be redox-regulated in higher eukaryotes (Bossis and Melchior, 2006; Kumar et al., 2007). Furthermore, mild oxidative stress triggers the formation of Uba1-Cdc34 disulfide complexes in *S. cerevisiae*, and Uba1 does not appear to form disulfides with other ubiquitin-specific E2s (our unpublished data). This indicates that only specific E1s and E2s are sensitive to redox regulation. Here, the Cdc34 orthologue, Ubc15, has been identified as a redox-sensitive E2 in *S. pombe*. To the best of our knowledge, this is the first time that redox regulation of an E2 has been shown to be conserved in eukaryotes. Furthermore, new techniques allowing the purification and identification of proteins containing sulfenic acid groups have indicated that mammalian Uba1 includes a redox-sensitive cysteine (Leonard et al., 2009). Thus, Uba1-E2 complex formation may be a conserved regulatory mechanism in eukaryotes. However, although Ubc12 is redox-regulated in mammalian cells (Kumar et al., 2007), Ubc12 oxidation could not be detected in *S. pombe*. It appears that the redox regulation of some E1s and E2s is conserved, whilst other enzymes are only redox-regulated in certain organisms. This could reflect organism-specific roles of the affected ubiquitin/Ubl conjugation pathway. For example, redox regulation of neddylation may affect downstream processes or proteins important for stress resistance in mammalian cells which are not found in yeast. Hence, there would be no evolutionary pressure to inhibit neddylation during oxidative stress in yeast. Indeed, microarray data suggests that the expression of *uba3*<sup>+</sup> and *ubc12*<sup>+</sup> is increased when *S. pombe* cells are treated with H<sub>2</sub>O<sub>2</sub> (Chen et al., 2008a), indicating that it is important to maintain neddylation during oxidative stress.

The amino acid sequences and tertiary structures of E2s appear to be important determinants of redox-sensitivity. Previous investigations of ubiquitin-specific E2s showed that these enzymes have similar tertiary structures, meaning that specific amino acid residues are positioned very close to the catalytic cysteine in many E2s (Tolbert et al., 2005). These amino acid residues include a conserved aspartate, which would be expected to increase the relative pKa of the catalytic cysteine and prevent oxidation (Tolbert et al., 2005). Significantly, budding yeast Cdc34 and fission yeast Ubc15 lack this aspartate residue, and both enzymes are redox-sensitive. Furthermore, there are two arginine residues upstream of the catalytic cysteine of Cdc34. These positively-charged, basic amino acid residues would be expected to stabilise thiolate anions nearby. Thus, the redox-sensitivity of an E2 could be predicted using the amino acid sequence of the protein. However, it is important to note that mammalian and yeast homologues of Ubc12 both lack the downstream aspartate residue found in many E2s, yet only mammalian Ubc12 appears to be redox-regulated. This could be explained by differences in the structure of Ubc12 homologues. While many E2s have similar core structures (van Wijk and Timmers, 2010), some E2s may have different tertiary structures which could position different amino acids near the catalytic cysteine residue. This would change the relative contributions of different amino acids to the control of the pKa of the catalytic cysteine residue. Therefore, although the amino acid sequences of E2s could allow us to predict which enzymes are redox-sensitive, it is also important to consider the structures of these enzymes when making such predictions.

Additionally, the transient microenvironments of catalytic cysteines may be an important factor controlling redox-sensitivity. As explained previously, E2s generally associate with an E1 after the E1 has formed a thioester with ubiquitin/Ubl. It is during this association that the catalytic cysteines of the E1 and E2 come into close proximity, and the E2 cysteine becomes deprotonated as part of the catalytic mechanism. However, the SUMO E2, Ubc9, can associate with the E1, Uba2, in the absence of SUMO (Wang et al., 2007). Coincidentally, the only published example of disulfide bond formation between the catalytic cysteines of an E1 and an E2 is in the mammalian SUMOylation pathway (Bossis and Melchior, 2006). It is possible that the environment of the cysteines is altered for long enough to allow oxidation and disulfide formation, which may not be the case for other pathways. Similarly, mammalian Ubc12 can form HMW complexes with proteins other than Uba3 following cysteine oxidation (Kumar et al., 2007). This may be due to Ubc12 associating with other proteins, again creating a

microenvironment favouring deprotonation of the catalytic cysteine residue. Interestingly, fission yeast Uba1 associates with other proteins under homeostatic conditions, and that these proteins produce a  $M_r$  shift expected when Uba1 associates with E2s. It is feasible that E2s with a strong affinity for E1 are most likely to become sequestered in E1-E2 disulfide complexes.

In conclusion, ubiquitin/Ubl conjugation can be redox-regulated via oxidation of the catalytic cysteine residues of specific E1s and E2s, and that this can have important implications for oxidative stress resistance in eukaryotes. Moreover, results in this chapter have shown that the redox-sensitivity of specific E2s may be conserved throughout evolution. In the future, it will be important to investigate the redox-sensitivity of other E2s to identify common features of enzymes sensitive to oxidation. For example, it would be interesting to investigate redox regulation of UbcP3 in *S. pombe*, as this E2 is 50% identical to Ubc15, and has a very similar amino acid sequence around the catalytic cysteine residue (Nielsen et al., 2002). Thus, Ubc15 and UbcP3 may share many structural properties that are predicted to confer redox-sensitivity. Moreover, structural characterisation and mutagenesis could be used to identify amino acid residues controlling redox-sensitivity of catalytic cysteines. This could allow us to predict which enzymes are sensitive to oxidative inactivation, and the resulting effects on downstream processes. However, in many cases, the downstream substrates of each E2 are unknown, preventing an investigation into how oxidative stress affects the ubiquitination of specific substrates. Thus, another priority for future research is to identify the E2s that facilitate the ubiquitination of key proteins involved in oxidative stress responses, and investigate whether these enzymes are redox-regulated.

## Extra References

- Azad, A.K., Tani, T., Shiki, N., Tsuneyoshi, S., Urushiyama, S., and Ohshima, Y. (1997). Isolation and molecular characterization of mRNA transport mutants in *Schizosaccharomyces pombe*. *Molecular biology of the cell* **8**, 825-841.
- Benito, J., Martin-Castellanos, C., and Moreno, S. (1998). Regulation of the G1 phase of the cell cycle by periodic stabilization and degradation of the p25<sup>rum1</sup> CDK inhibitor. *The EMBO journal* **17**, 482-497.
- Bossis, G., and Melchior, F. (2006). Regulation of SUMOylation by reversible oxidation of SUMO conjugating enzymes. *Molecular cell* **21**, 349-357.
- Byers, B., and Goetsch, L. (1974). Duplication of spindle plaques and integration of the yeast cell cycle. *Cold Spring Harbor symposia on quantitative biology* **38**, 123-131.
- Craven, R.A., Griffiths, D.J., Sheldrick, K.S., Randall, R.E., Hagan, I.M., and Carr, A.M. (1998). Vectors for the expression of tagged proteins in *Schizosaccharomyces pombe*. *Gene* **221**, 59-68.
- Day, A.M., Brown, J.D., Taylor, S.R., Rand, J.D., Morgan, B.A., and Veal, E.A. (2012). Inactivation of a peroxiredoxin by hydrogen peroxide is critical for thioredoxin-mediated repair of oxidized proteins and cell survival. *Molecular cell* **45**, 398-408.
- Glotzer, M., Murray, A.W., and Kirschner, M.W. (1991). Cyclin is degraded by the ubiquitin pathway. *Nature* **349**, 132-138.
- Goehl, M.G., Yochem, J., Jentsch, S., McGrath, J.P., Varshavsky, A., and Byers, B. (1988). The yeast cell cycle gene CDC34 encodes a ubiquitin-conjugating enzyme. *Science* **241**, 1331-1335.
- Herrmann, J., Lerman, L.O., and Lerman, A. (2007). Ubiquitin and ubiquitin-like proteins in protein regulation. *Circulation research* **100**, 1276-1291.
- Hochstrasser, M. (2000). Evolution and function of ubiquitin-like protein-conjugation systems. *Nature cell biology* **2**, E153-157.



Hochstrasser, M. (2009). Origin and function of ubiquitin-like proteins. *Nature* 458, 422-429.

Jahngen-Hodge, J., Obin, M.S., Gong, X., Shang, F., Nowell, T.R., Jr., Gong, J., Abasi, H., Blumberg, J., and Taylor, A. (1997). Regulation of ubiquitin-conjugating enzymes by glutathione following oxidative stress. *The Journal of biological chemistry* 272, 28218-28226.

Kerscher, O., Felberbaum, R., and Hochstrasser, M. (2006). Modification of proteins by ubiquitin and ubiquitin-like proteins. *Annual review of cell and developmental biology* 22, 159-180.

King, R.W., Deshaies, R.J., Peters, J.M., and Kirschner, M.W. (1996). How proteolysis drives the cell cycle. *Science* 274, 1652-1659.

Kumar, A., Wu, H., Collier-Hyams, L.S., Hansen, J.M., Li, T., Yamoah, K., Pan, Z.Q., Jones, D.P., and Neish, A.S. (2007). Commensal bacteria modulate cullin-dependent signaling via generation of reactive oxygen species. *The EMBO journal* 26, 4457-4466.

Labib, K., and Moreno, S. (1996). rum1: a CDK inhibitor regulating G1 progression in fission yeast. *Trends in cell biology* 6, 62-66.

Leonard, S.E., Reddie, K.G., and Carroll, K.S. (2009). Mining the thiol proteome for sulfenic acid modifications reveals new targets for oxidation in cells. *ACS chemical biology* 4, 783-799.

Mathias, N., Steussy, C.N., and Goebel, M.G. (1998). An essential domain within Cdc34p is required for binding to a complex containing Cdc4p and Cdc53p in *Saccharomyces cerevisiae*. *The Journal of biological chemistry* 273, 4040-4045.

Nielsen, I.S., Nielsen, O., Murray, J.M., and Thon, G. (2002). The fission yeast ubiquitin-conjugating enzymes UbcP3, Ubc15, and Rhp6 affect transcriptional silencing of the mating-type region. *Eukaryotic cell* 1, 613-625.

Osaka, F., Saeki, M., Katayama, S., Aida, N., Toh, E.A., Kominami, K., Toda, T., Suzuki, T., Chiba, T., Tanaka, K., *et al.* (2000). Covalent modifier NEDD8 is essential for SCF ubiquitin-ligase in fission yeast. *The EMBO journal* 19, 3475-3484.

Pagano, M. (1997). Cell cycle regulation by the ubiquitin pathway. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 11, 1067-1075.

Petroski, M.D., and Deshaies, R.J. (2005). Mechanism of lysine 48-linked ubiquitin-chain synthesis by the cullin-RING ubiquitin-ligase complex SCF-Cdc34. *Cell* 123, 1107-1120.

Sanchez-Diaz, A., Gonzalez, I., Arellano, M., and Moreno, S. (1998). The Cdk inhibitors p25rum1 and p40SIC1 are functional homologues that play similar roles in the regulation of the cell cycle in fission and budding yeast. *Journal of cell science* 111 ( Pt 6), 843-851.

Schwob, E., Bohm, T., Mendenhall, M.D., and Nasmyth, K. (1994). The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in *S. cerevisiae*. *Cell* 79, 233-244.

Tolbert, B.S., Tajc, S.G., Webb, H., Snyder, J., Nielsen, J.E., Miller, B.L., and Basavappa, R. (2005). The active site cysteine of ubiquitin-conjugating enzymes has a significantly elevated pKa: functional implications. *Biochemistry* 44, 16385-16391.

van Wijk, S.J., and Timmers, H.T. (2010). The family of ubiquitin-conjugating enzymes (E2s): deciding between life and death of proteins. *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 24, 981-993.

Wang, J., Hu, W., Cai, S., Lee, B., Song, J., and Chen, Y. (2007). The intrinsic affinity between E2 and the Cys domain of E1 in ubiquitin-like modifications. *Molecular cell* 27, 228-237.

Welchman, R.L., Gordon, C., and Mayer, R.J. (2005). Ubiquitin and ubiquitin-like proteins as multifunctional signals. *Nature reviews Molecular cell biology* 6, 599-609.

## Chapter 4. Regulation and roles of urmylation in fission yeast stress responses.

### 4.1. Introduction

While ubiquitin/Ubl conjugation can be inhibited during oxidative stress in eukaryotes, many of these pathways are not inhibited during oxidative stress, likely because the structures of specific enzymes have evolved such that their catalytic cysteine residues are insensitive to oxidation. Indeed, urmylation is actually increased during oxidative stress in both *S. cerevisiae* and mammalian cells (Goehring et al., 2003a; Van der Veen et al., 2011). While the molecular basis for this remains unknown, it is possible that protein urmylation is important for responses to oxidative stress. In support of this idea, *Urm1* cells display increased sensitivity to oxidative stress in *S. cerevisiae* (Furukawa et al., 2000; Goehring et al., 2003a). However, it is unclear whether urmylation is also important for resistance to oxidative stress in other organisms.

The highly-conserved Ubl Urm1 was initially identified in *S. cerevisiae* by homology to the prokaryotic sulphur carriers MoeD and ThiS (Furukawa et al., 2000). The E1, Uba4, was identified as an Urm1-interacting protein using a yeast two-hybrid assay (Furukawa et al., 2000). Similar to other UbIs, Urm1 is conjugated to lysine residues of specific proteins (Goehring et al., 2003b; Van der Veen et al., 2011), implying that a second enzyme may be required to confer specificity. However, no Urm1-specific E2 has yet been identified. Hence, it is also possible that Uba4 interacts with unknown adaptor proteins to target Urm1 conjugation to particular substrates. Although a limited number of urmylated proteins have been identified, the effect of Urm1 conjugation on protein function remains unknown, and no conserved Urm1 substrates have yet been identified.

As well as its role in the post-translational modification of proteins, Urm1 also functions as a sulphur carrier to facilitate tRNA thiolation in eukaryotes (Nakai et al.,

2008; Leidel et al., 2009). The wobble uridines of tRNA<sup>Lys</sup><sub>UUU</sub>, tRNA<sup>Glu</sup><sub>UUC</sub> and tRNA<sup>Gln</sup><sub>UUG</sub> are modified by mcm groups and sulphur, which is important for interactions with the ribosome and for translational fidelity (Ashraf et al., 1999; Yarian et al., 2002). Unlike other UbIs, Urm1 activation involves thiocarboxylation of the C-terminus by Uba4 (**Fig. 1.2**), allowing Urm1 to carry sulphur. Thus, Urm1 may represent an evolutionary link between prokaryotic sulphur transfer and eukaryotic post-translational modification pathways.

It is clear that urmylation has a role in stress responses, and may itself be regulated during oxidative stress. However, it is unclear how urmylation is regulated by oxidative stress. Furthermore, urmylation has never been shown to have conserved roles in oxidative stress resistance. Hence, urmylation was investigated in *S. pombe* to determine whether this pathway has conserved roles in stress resistance, as well as to investigate redox regulation of urmylation and to identify conserved Urm1 substrates which may have roles in oxidative stress responses.

## **4.2. The roles of urmylation in oxidative stress responses in *S. pombe*.**

### **4.2.1. Dysfunctional urmylation causes sensitivity to oxidative and heat stresses in *S. pombe*.**

As described in Chapter 3, dysfunctional ubiquitination, SUMOylation or neddylation causes full or partial loss of viability in eukaryotes. In contrast, dysfunctional urmylation does not affect the viability of *S. cerevisiae* cells (Furukawa et al., 2000). Thus, a  $\Delta$ urm1 strain may also be used to investigate how dysfunctional urmylation affects stress resistance in *S. pombe*. Firstly, fission yeast homologues of Urm1 and Uba4 were identified by BLAST search of the *S. pombe* proteome, using the amino acid sequences of *S. cerevisiae* Urm1 and Uba4 (**Fig. 4.1**). The fission yeast homologue of Urm1 is a protein with a M<sub>r</sub> of ~11kDa, and is encoded by a gene on chromosome III (*SPCC548.04*) which will be referred to as *urm1*<sup>+</sup>. Fission yeast Uba4 has a M<sub>r</sub> of

## A

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ScUrm1  MVNVKVEFLGGLD AIFGKQRVH KIKMDKEDPVTVGDLIDHIVSTMINNPNDVSIFIEDDS
SpUrm1  -MAIKVELLGGLDLLFNKQKALSLSLSNLGSTKLGLIDYMAQII EK-PSQKDLFILNGT
      : :***:***** : * ** : : : : : * ***: : : : * : : ** : :
ScUrm1  IRPGIITLINDTDWELEGEKDYILEDGDIISFTSTLHGG
SpUrm1  VRPGIIVLVNDQDWELLEKEEYNLEEGDEVVFVSTLHGG
      :***** *:* ** * : : * **:* : * *****

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## B

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ScUba4  MNDYHLEDTTSELEALRL ENAQLREQLAKREDSSRDYPLSLEEYQRYGRQMIVEETGGVA
SpUba4  -----MNPQEKIICSSNGLELSLDEYSRYGRQMLLSEIG-LP
      : * ** ***:* *****: * * :
ScUba4  GQVKLKNTKVLVVGAGGLGCPALPYLAGAGVGQIGIVDNDVETS NLHRQVLHDSSRVGM
SpUba4  GQLSLKRSSVLVIGAGGLGCPAMQYLVAAGIGTLGIMDGDVVDKSNLHRQIIHSTSKQGM
      **: ** : ***:*****: ** **:* **: * ***: *****: : * : **
ScUba4  LKCESARQYITKLNPHINVVTYPVRLNSSNAFDIFKGYNIILDCTDSPLTRYLVS DVAVN
SpUba4  HKAISAKQFLEDLNPVNIINTYLEFASASNLSIIEQYDVVLDCTDNQYTRYLISDTCVL
      * **:* : : ***: : ** : ** * *: : * :***** *****:* *
ScUba4  LGITVVSASGLGTEGQLTILNFNNIGPCYRCFYPTPPPPNAVTSCEGGVIGPCIGLVGT
SpUba4  LGRPLVSASALKLEGQLCIYNYCNG-PCYRCMFNPPTP--VVASCAKSGILGPVVGTMGT
      ** :***** * ***** * *: * *****: * * * *: ** : *: ** : **
ScUba4  MMAVETLKLILGIYTN--ENFSPFLMLYSGFPQQSLRTFKMRGRQEKCLCCGKNRTITKE
SpUba4  MQALETVKLILHINGIKDQFDPYMLLFHAFKVPQWKHIRIRPRQQSCKACGPNKMLSRE
      * *:***:***** :*: * :*: * : :*: * ** * :*: *
ScUba4  AIEKGEINYELFCGARNYNVCEPDERISVDAFQRIYKDDEFLAKHIFLDVRPSHHYEISH
SpUba4  FMESSPKEYTTICDYVPTLSKQLAPIRRISALD-LKNLIETSPHITFLDVREFPVQFGICR
      : * : * : : : : * : ***** : * :
ScUba4  FPEAVNIPIKNLRDMNGDLKKLQEKLPSEKDSNIVILCRYGNDSQLATRLKDKFGFSN
SpUba4  LPLFKNIPLSEVDSLQN-----LSGKVCVICRSGNTSQTAVRKLQELNPQAD
      : * ***: : : : : : : ** * * * * *: : :
ScUba4  VRDVRGGYFKYID DIDQTIPKY
SpUba4  IFDVVAGLKGWSTEVDNPFPLY
      : ** * : : * : *

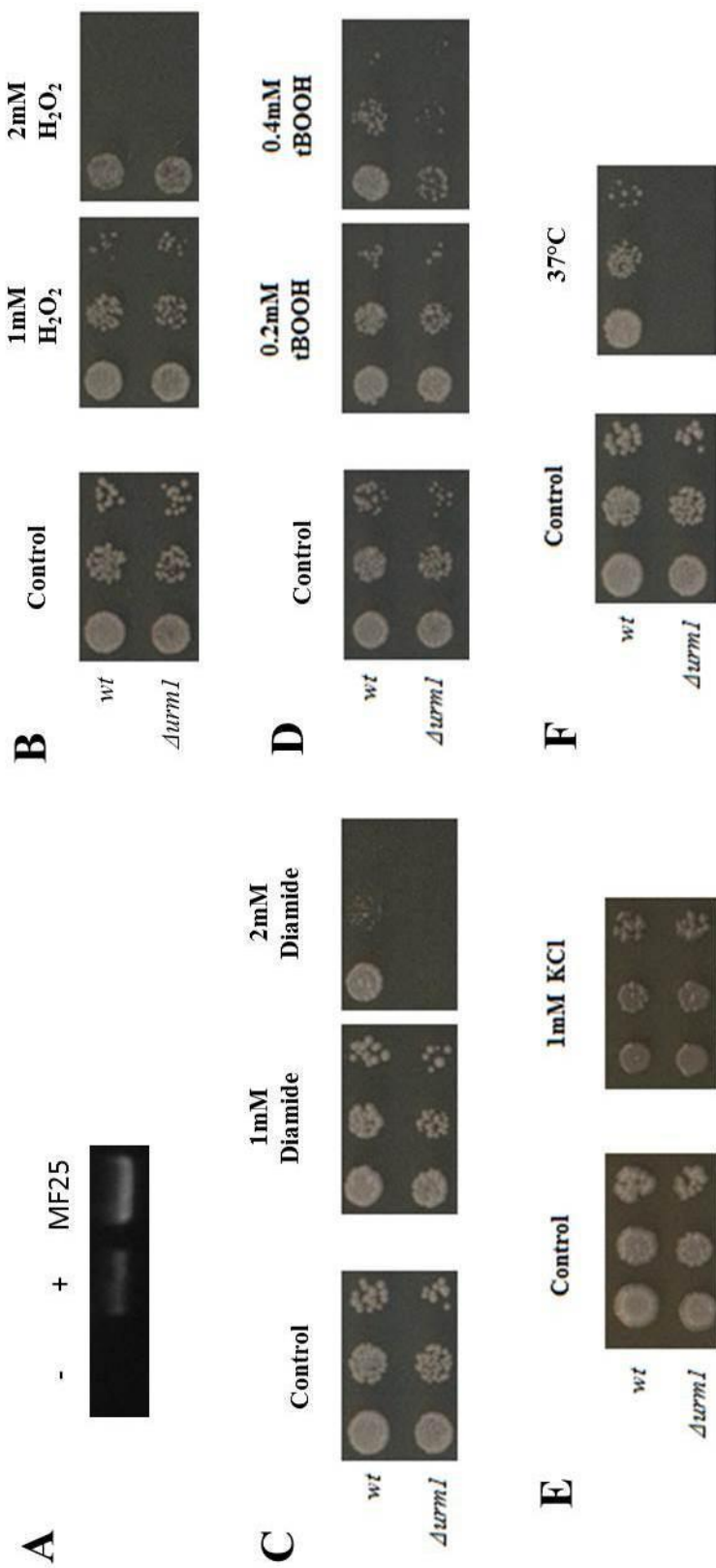
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**Figure 4.1. Identification of *S. pombe* homologues of Urm1 and Uba4.**

Fission yeast homologues of *S. cerevisiae* (Sc) Urm1 and Uba4 were identified by BLAST search of the *S. pombe* (Sp) proteome, with 44% and 40% identity, respectively. Amino acid sequence alignments for (A) Urm1 and (B) Uba4 are shown above. Identical residues are indicated by asterisks, and similar residues are indicated by colons.

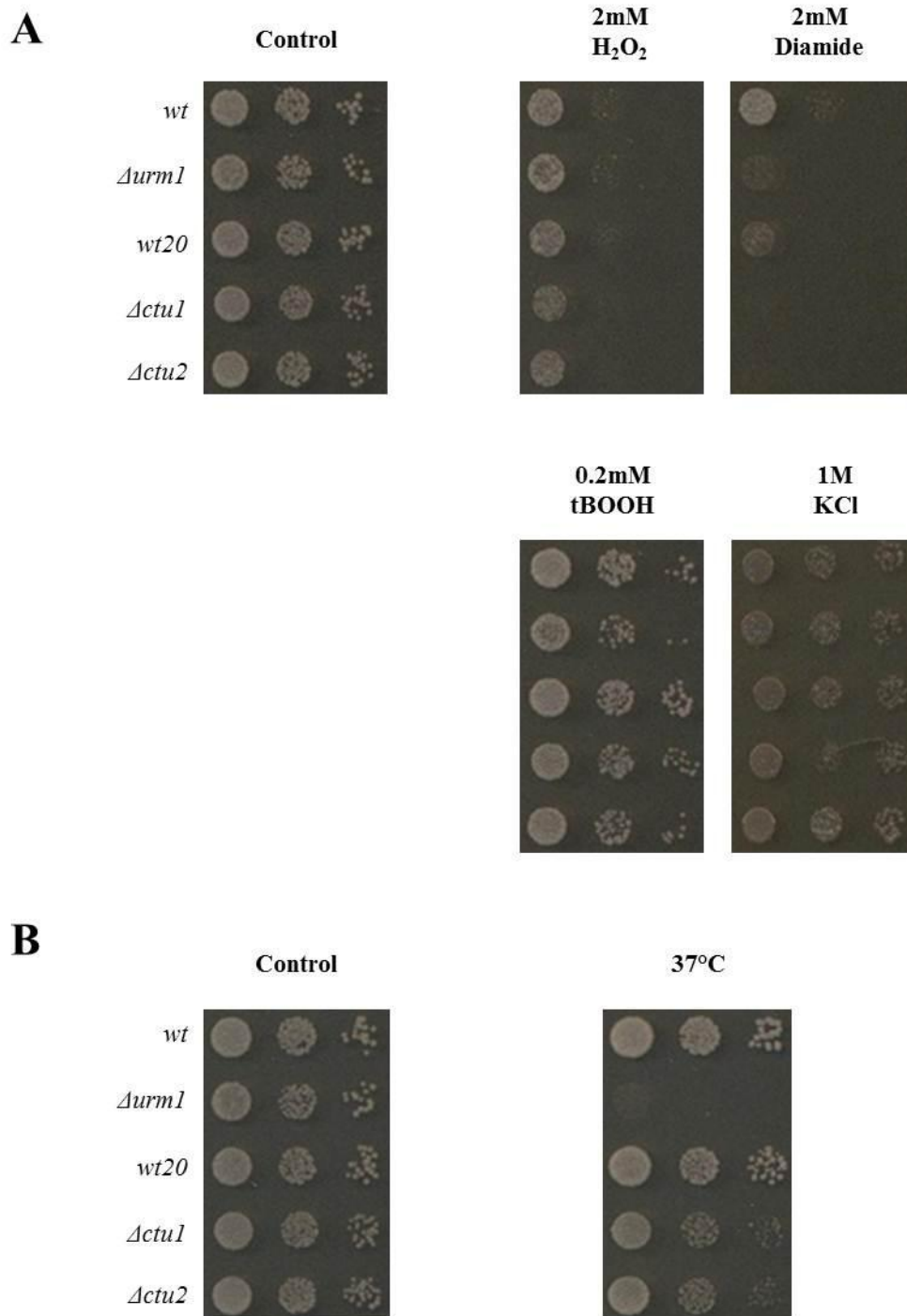
~45kDa, and is encoded by a gene on chromosome I (*SPAC2G11.10c*), which is hereafter referred to as *uba4*<sup>+</sup>. A Bioneer deletion library includes a strain (BG\_4808) with a *KanMX6* cassette at the *urm1*<sup>+</sup> locus. Thus, the strategy outlined in **Fig. 3.10A** was used to construct a *Δurm1* strain using our own strain backgrounds. Incorporation of *KanMX6* at the *urm1*<sup>+</sup> locus was confirmed by PCR (**Fig. 4.2A**). To investigate whether urmylation is involved in stress responses, *Δurm1* cells were exposed to a range of stress conditions. Cells lacking Urm1 displayed increased sensitivity to oxidative stress induced by diamide and *t*-BOOH, but not H<sub>2</sub>O<sub>2</sub> (**Fig. 4.2B-D**), or to osmotic stress induced by KCl (**Fig. 4.2E**). Additionally, *Δurm1* cells were highly sensitive to growth at 37°C, in contrast to wild-type cells (**Fig. 4.2F**). Given that urmylation also appears to be important for resistance to oxidative and heat stresses in *S. cerevisiae* (Furukawa et al., 2000; Goehring et al., 2003a), these data indicate that urmylation has roles in stress resistance that are highly conserved in eukaryotes. However, budding yeast *Δurm1* cells do not display increased sensitivity to *t*-BOOH (Goehring et al., 2003a), suggesting that urmylation may also have species-specific roles in protecting against oxidative stress.

Although urmylation is important for resistance to heat and oxidative stress in *S. pombe*, it was unclear whether *Δurm1* cells display increased sensitivity to stress due to loss of protein modification or loss of sulphur transfer to tRNAs. In *S. cerevisiae*, the transfer of sulphur from thiocarboxylated Urm1 to tRNAs requires the ATPases Ncs6 and Ncs2 (Leidel et al., 2009). Fission yeast homologues of Ncs6 and Ncs2 are encoded by *ctu1*<sup>+</sup> and *ctu2*<sup>+</sup>, respectively, and these genes are essential for tRNA thiolation (Dewez et al., 2008). Hence, to investigate whether the stress sensitivities of *Δurm1* are due to loss of protein modification or loss of sulphur transfer, the phenotypes of *Δurm1* cells were compared to those of *Δctu1* and *Δctu2* cells (gift from D. Hermand). Significantly, *Δurm1*, *Δctu1* and *Δctu2* cells displayed similar sensitivity to H<sub>2</sub>O<sub>2</sub>, diamide, *t*-BOOH and osmotic stress induced by KCl (**Fig. 4.3A**). In addition, *Δctu1* and *Δctu2* cells were more temperature-sensitive than wild-type cells, in agreement with previous findings (Dewez et al., 2008). However, *Δurm1* cells were far more sensitive to heat than *Δctu1* and *Δctu2* cells (**Fig. 4.3B**). Due to the *Δurm1* and *Δctu1/Δctu2* strains being constructed in different strain backgrounds, two different wild-type strains were used. Despite this, the stress sensitivities of these two strains were generally very similar. Taken together, these results suggest that *Δurm1* cells are sensitive to diamide because sulphur transfer to tRNAs is prevented. This may also be the case in *S. cerevisiae*



**Figure 4.2. Fission yeast lacking the urmylation pathway display increased sensitivity to diamide, *t*-BOOH and heat, but not to  $H_2O_2$  or osmotic stress induced by KCl.**

**A.** A *Δurm1* strain was constructed using the technique described in Fig. 3.10A, using the oligonucleotide primers *Urm1delf* and *Urm1delr* (see **Table 2.2**) and gDNA from BG\_4808 as a template for PCR. To confirm *Δurm1* strain construction, gDNA was extracted from a G-418-resistant colony (MF25), as well as wild-type (CHP429; -) and Bioneer *Δurm1* (BG\_4808; +) cells. PCR was performed using these DNA extracts as templates, using the forward primer *Urm1delf* and the reverse primer *KanMX6delchk*, which binds to a site within *KanMX6* (see **Table 2.2**). PCR products were analysed by agarose gel electrophoresis, and a PCR product of 1.2kbp confirmed strain construction. **B-F.** To investigate sensitivity to oxidative and osmotic stresses, ten-fold serial dilutions of mid-log phase growing wild-type (wt; CHP429) and *Δurm1* (MF25) cells were spotted onto YE5S plates containing the indicated concentrations of **(B)**  $H_2O_2$ , **(C)** diamide, **(D)** *t*-BOOH and **(E)** KCl, and incubated at 30°C for three days. To investigate sensitivity to heat **(F)**, a YE5S plate was incubated at 37°C for three days. The above results are representative of three independent experiments.



**Figure 4.3. Dysfunctional urmylation may cause oxidative stress sensitivity due to loss of sulphur transfer, whilst heat sensitivity may be due to loss of both sulphur transfer and protein urmylation.**

To investigate sensitivity to different stress conditions, ten-fold serial dilutions of mid-log phase growing wild-type (*wt*; CHP429),  $\Delta urm1$  (MF25), wild-type (*wt20*; DH20),  $\Delta ctu1$  (DH252) and  $\Delta ctu2$  (DH237) cells were spotted onto YE5S plates (**A**) containing the indicated concentrations of  $H_2O_2$ , diamide, *t*-BOOH and KCl, and incubated at 30°C for three days. To investigate sensitivity to heat (**B**), a YE5S plate was incubated at 37°C for three days. These experiments were repeated, and gave the same results.

(Leidel et al., 2009). In contrast, the fact that *Δurm1* cells are more sensitive to heat than *Δctu1* and *Δctu2* cells indicates that the temperature sensitivity of *Δurm1* cells results from the loss of both sulphur transfer and protein urmylation.

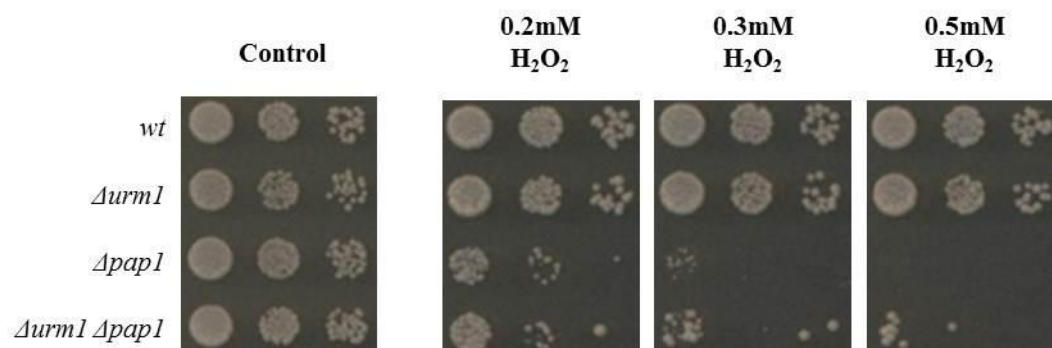
#### **4.2.2. Dysfunctional urmylation slightly decreases the H<sub>2</sub>O<sub>2</sub>-sensitivity of *Δpap1* cells.**

The sensitivity of *Δurm1* cells to oxidative and heat stresses suggests that the urmylation pathway may regulate stress response mechanisms in *S. pombe*. Although the above results indicate that Urm1 is not required for resistance to H<sub>2</sub>O<sub>2</sub>, a functional urmylation pathway is important for survival when cells are exposed to other oxidative stress agents. Hence, the genetic relationship between the urmylation pathway and oxidative stress response mechanisms was investigated. As outlined in Section 1.3, oxidative stress responses are very well-characterised in *S. pombe*. The transcription factor Pap1 is rapidly activated by low concentrations of H<sub>2</sub>O<sub>2</sub> (Quinn et al., 2002), and regulates gene expression during oxidative stress (Madrid et al., 2004; Toone et al., 1998). Hence, the genetic relationship between *urm1*<sup>+</sup> and *pap1*<sup>+</sup> was investigated. A *Δurm1Δpap1* strain was constructed via genetic cross (see Section 2.2.3) between the MF25 and JP178 strains and sensitivity to H<sub>2</sub>O<sub>2</sub> was examined. As expected, *Δpap1* cells displayed increased sensitivity to H<sub>2</sub>O<sub>2</sub>, relative to wild-type cells (**Fig. 4.4**). However, *Δurm1Δpap1* cells were slightly less sensitive to H<sub>2</sub>O<sub>2</sub> than *Δpap1* cells (**Fig. 4.4**), suggesting that dysfunctional urmylation partially rescues the H<sub>2</sub>O<sub>2</sub>-sensitivity of cells lacking Pap1.

#### **4.2.3. Atf1 protein levels are increased in *Δurm1* cells, relative to wild-type.**

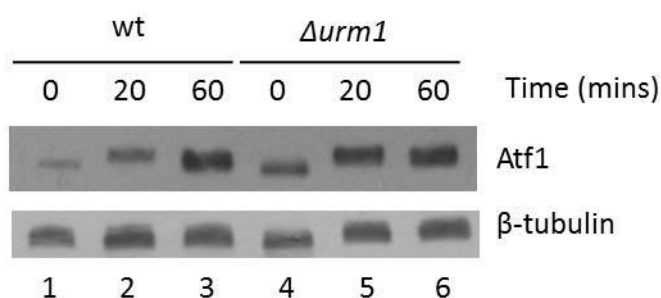
It was possible that other oxidative stress response mechanisms are upregulated in *Δurm1* cells, and may compensate for loss of Pap1. The fission yeast SAPK Sty1 is activated in response to many stimuli including oxidative, heat and osmotic stresses





**Figure 4.4. Loss of the urmylation pathway slightly decreases the oxidative stress sensitivity of *Δpap1*.**

Ten-fold serial dilutions of mid-log phase growing wild-type (CHP429), *Δurm1* (MF25), *Δpap1* (JP178) and *Δurm1 Δpap1* (MF75) cells were spotted onto YE5S plates including the indicated concentrations of H<sub>2</sub>O<sub>2</sub>, and incubated at 30°C for three days. The above result is representative of three experiments.



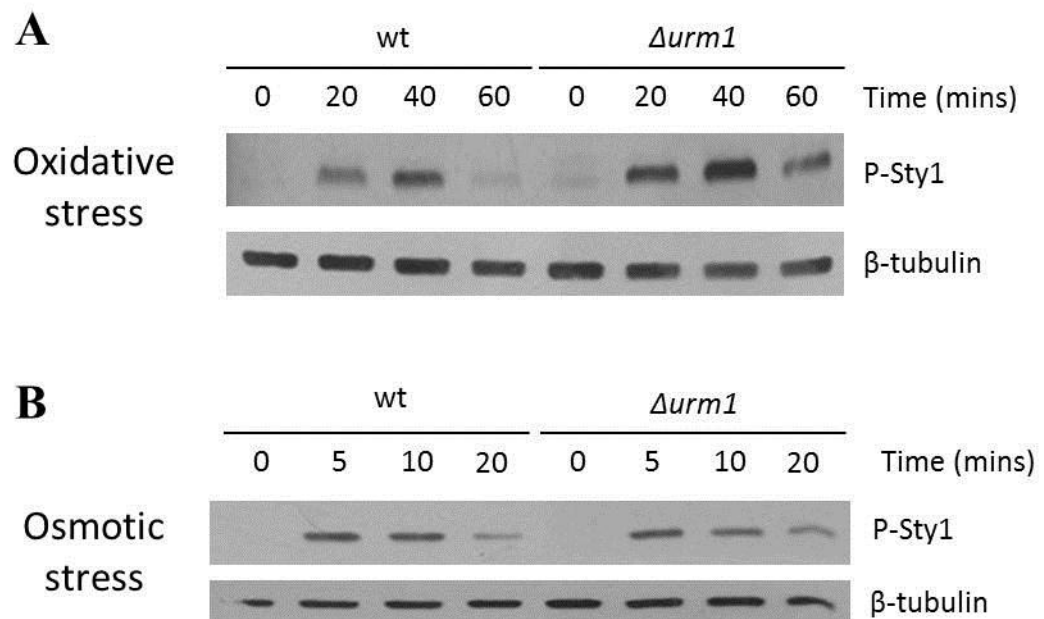
**Figure 4.5. Loss of the urmylation pathway increases levels of Atf1, and may increase Atf1 phosphorylation during oxidative stress.**

Wild-type (wt; JP180) and *Δurm1* (MF84) cells expressing 6HisHA-tagged Atf1 were exposed to 1mM H<sub>2</sub>O<sub>2</sub>, and cells harvested after 0, 20 and 60 minutes. Protein extracts were prepared by TCA lysis. Atf1 levels and phosphorylation were investigated by SDS-PAGE and western blotting using anti-HA antibodies. β-tubulin levels are shown as a loading control. The above result is representative of three experiments.

(Degols et al., 1996; Kato et al., 1996; Millar et al., 1995; Shiozaki and Russell, 1995; Toone et al., 1998). Sty1 translocates into the nucleus and activates the transcription factor Atf1 (Wilkinson et al., 1996), which controls the expression of genes involved in responses to environmental stress. Furthermore, a second SAPK, Pmk1, also phosphorylates Atf1 (Takada et al., 2007) and promotes Atf1-dependent gene expression during oxidative stress (Chen et al., 2008). Thus, Atf1 is another transcription factor involved in oxidative stress responses, and could be regulated by urmylation. To investigate this possibility, a *Δurm1* strain expressing 6HisHA-tagged Atf1 was obtained by genetic cross between the MF44 and JP180 strains, treated with H<sub>2</sub>O<sub>2</sub>, and Atf1 mobility through SDS-PAGE gels was analysed by western blotting. Although there is some indication that Atf1 phosphorylation is increased and prolonged during H<sub>2</sub>O<sub>2</sub> exposure in *Δurm1* cells (**Fig. 4.5**), it is difficult to say this conclusively. However, basal Atf1 levels are clearly increased in *Δurm1* cells (**Fig. 4.5, compare lanes 1 and 4**), implying a role for Urm1 in regulating Atf1 stability. Both of these possibilities require further investigation. Nevertheless, it remains possible that *Δurm1Δpap1* cells are slightly more resistant to H<sub>2</sub>O<sub>2</sub> than *Δpap1* cells as a result of effects on Atf1.

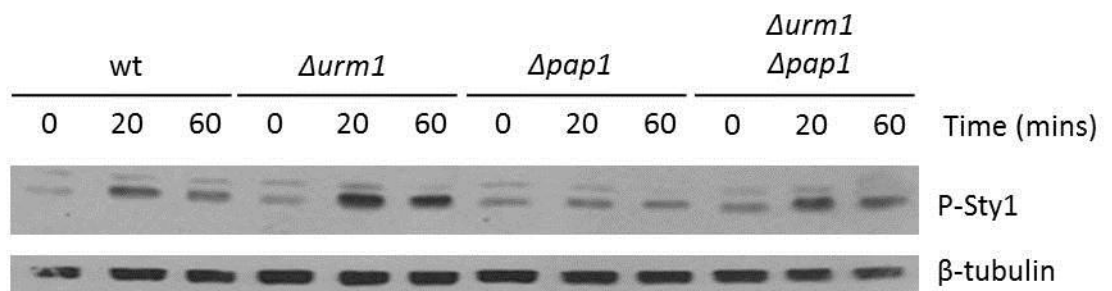
#### 4.2.4. H<sub>2</sub>O<sub>2</sub>-induced Sty1 phosphorylation is increased in *Δurm1* cells.

As outlined above, Sty1 activates Atf1 in response to various stress conditions. Thus, it was possible that urmylation somehow regulates Atf1 via the upstream Sty1 pathway. Hence, to investigate the effects of dysfunctional urmylation on Sty1 activation, wild-type and *Δurm1* cells were treated with H<sub>2</sub>O<sub>2</sub> or KCl. Protein extracts were prepared, Sty1 phosphorylation was examined by western blotting. Dysfunctional urmylation has no effect on basal Sty1 phosphorylation (**Fig. 4.6**). However, the oxidative stress-induced activation of Sty1 is increased and prolonged in *Δurm1* cells (**Fig. 4.6A**). In contrast, dysfunctional urmylation had no effect on the osmotic stress-induced activation of Sty1 (**Fig. 4.6B**). These results indicate that Urm1 limits the activation of Sty1 when cells are exposed to H<sub>2</sub>O<sub>2</sub>, but not during osmotic stress. It is therefore possible that dysfunctional urmylation causes *Δpap1* cells to become slightly less sensitive to H<sub>2</sub>O<sub>2</sub> due to the increased activation of Sty1, which may partially compensate for the loss of Pap1. To investigate this further, Sty1 activation was



**Figure 4.6. Loss of the urmylation pathway increases Sty1 activation in response to oxidative stress, but not osmotic stress.**

Wild-type (wt; CHP429) and *Δurm1* (MF25) cells were treated with (A) 1mM H<sub>2</sub>O<sub>2</sub> or (B) 0.6M KCl. Cells were harvested at the time-points shown, and extracts prepared according to the MAPK assay protocol outlined previously. Levels of phosphorylated Sty1 (P-Sty1) were analysed by western blotting, using anti-phospho-p38 antibodies. β-tubulin levels are shown as a loading control. The above results are representative of three experiments.



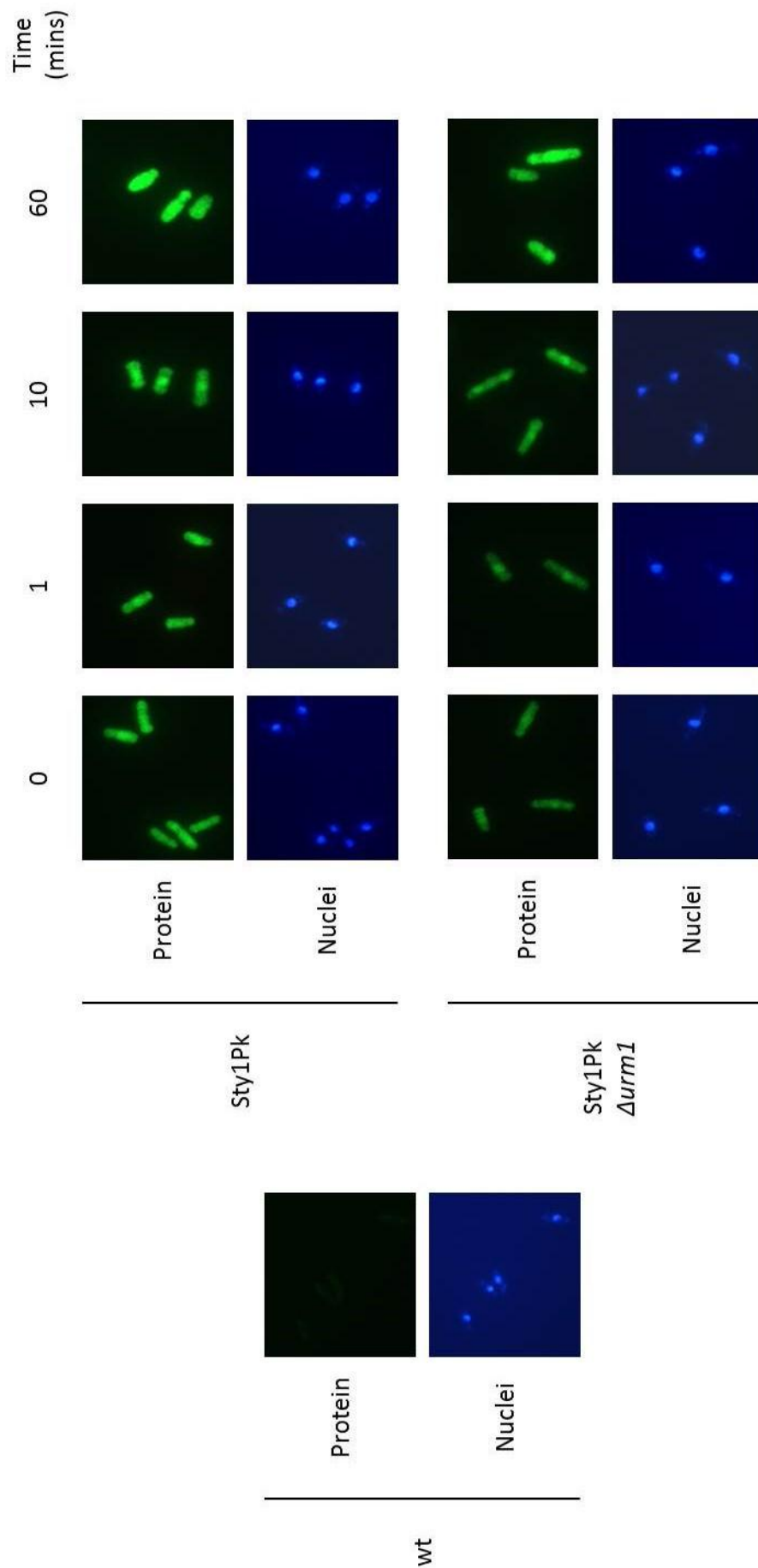
**Figure 4.7. The increased activity of Sty1 in *Δurm1* cells may partially compensate for loss of Pap1.**

Wild-type (wt; CHP429), *Δurm1* (MF25), *Δpap1* (JP178) and *Δurm1 Δpap1* (MF75) cells were exposed to 1mM H<sub>2</sub>O<sub>2</sub>, and cells harvested at the time-points shown. Protein extracts were prepared using the MAPK assay protocol. Levels of phosphorylated Sty1 (P-Sty1) were examined by western blotting, using anti-phospho-p38 antibodies. β-tubulin levels are shown as a loading control. This result is representative of three different experiments.

examined in *Δpap1* and *Δurm1Δpap1* cells. Curiously, the H<sub>2</sub>O<sub>2</sub>-induced activation of Sty1 was decreased in *Δpap1* cells, relative to wild-type (**Fig. 4.7**). However, Sty1 activation was higher in *Δurm1Δpap1* cells than in *Δpap1* cells (**Fig. 4.7**). This supports the hypothesis that increased Sty1 activity partially compensates for loss of Pap1 in *Δurm1Δpap1* cells, and may explain the prior observation that *Δurm1Δpap1* cells are less sensitive to H<sub>2</sub>O<sub>2</sub> than *Δpap1* cells.

Following activation, Sty1 translocates into the nucleus and activates Atf1. Given that oxidative stress-induced Sty1 phosphorylation is increased in *Δurm1* cells, it was possible that the nuclear accumulation of Sty1 is also increased. To investigate this possibility, a *Δurm1* strain expressing Pk epitope-tagged Sty1 from the chromosomal locus was generated by genetic cross between the MF44 and AD13 strains, and Sty1 was activated by treating cells with H<sub>2</sub>O<sub>2</sub>. Cells were fixed using paraformaldehyde, and Sty1 localisation was analysed by indirect immunofluorescence (**Fig. 4.8**). Under homeostatic conditions, Sty1 is present throughout the cell. When cells are treated with H<sub>2</sub>O<sub>2</sub>, Sty1 gradually accumulates in the nucleus. Sty1 localisation appears to be unaffected in *Δurm1* cells. However, the possibility that Sty1 nuclear accumulation is increased in *Δurm1* cells cannot be ruled out. When using indirect immunofluorescence, Sty1 levels cannot be quantified, and Sty1 localisation can only be observed at specific points in time. Ideally, to analyse Sty1 localisation more accurately, levels of nuclear Sty1 should be quantified, and Sty1 localisation could be monitored in live cells using a fluorophore-tagged form of the protein.

Although Sty1 phosphorylation is clearly regulated by Urm1, the underlying mechanism remains unclear. As described in Section 1.3.2, Sty1 is activated by different mechanisms in response to different stress conditions. These mechanisms include activation of the upstream MAPK cascade and downregulation of Sty1-specific phosphatases. Wis1 is the MAPKK functioning upstream of Sty1, and the phosphomimetic Wis1DD is a constitutively-active form of the enzyme. Sty1 activation is dramatically increased in Wis1DD cells under homeostatic conditions, and oxidative stress does not increase Sty1 activation any further (Nguyen and Shiozaki, 1999; Shiozaki et al., 1998). Thus, if Urm1 regulates Sty1 via upstream kinases, there would be no difference in Sty1 phosphorylation in Wis1DD and *Δurm1* Wis1DD cells during oxidative stress. However, if Urm1 regulates Sty1 activation independently of Wis1, Sty1 phosphorylation would be increased in *Δurm1* Wis1DD cells compared to



**Figure 4.8. Dysfunctional urmylation did not cause a detectable effect on the stress-induced nuclear accumulation of Sty1.**

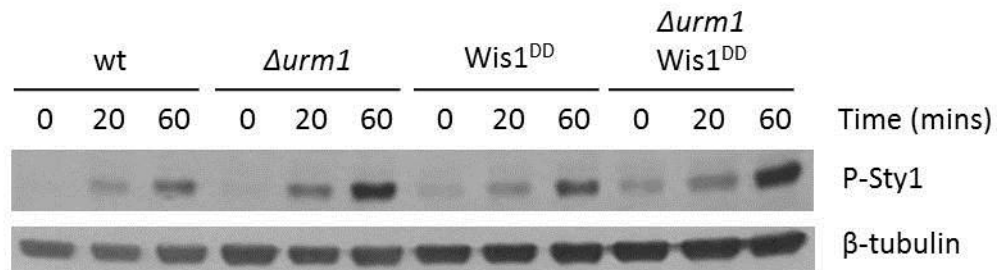
To investigate Sty1 localization, mid-log phase Sty1Pk (AD13) and Sty1Pk  $\Delta urm1$  (MF45) cells were treated with 1mM H<sub>2</sub>O<sub>2</sub> for the times indicated, and cells were harvested and fixed with 3.7% paraformaldehyde. Wild-type (wt; CHP429) cells were also harvested and fixed, as a control. Sty1Pk was visualised by indirect immunofluorescence using anti-Pk antibodies and Alexa Fluor<sup>®</sup>-conjugated anti-mouse antibodies. Nuclei were visualised by DAPI staining. This experiment was repeated and gave the same result each time.

Wis1DD cells. To investigate whether Urm1 regulates Sty1 in a Wis1-dependent manner, a *Δurm1* Wis1DD strain was obtained by genetic cross between the MF25 and MF88 strains, and Sty1 phosphorylation was analysed in *Δurm1*, Wis1DD and *Δurm1* Wis1DD cells following H<sub>2</sub>O<sub>2</sub> treatment. Levels of phosphorylated Sty1 were higher in *Δurm1* Wis1DD cells than in Wis1DD cells (**Fig. 4.9**), indicating that urmylation regulates Sty1 activation independently of the MAPK cascade. Interestingly, these results contradict previous reports that Sty1 phosphorylation is not increased when cells expressing Wis1DD are exposed to H<sub>2</sub>O<sub>2</sub> (Nguyen and Shiozaki, 1999; Shiozaki et al., 1998). As expected, basal Sty1 phosphorylation was increased in Wis1DD cells (**Fig. 4.9**). However, Sty1 phosphorylation was further increased by H<sub>2</sub>O<sub>2</sub> treatment. This suggests that H<sub>2</sub>O<sub>2</sub> may activate Sty1 via both Wis1-dependent and Wis1-independent mechanisms. In support of this, Sty1 phosphorylation is slightly increased when cells are exposed to 0.2mM H<sub>2</sub>O<sub>2</sub>, whereas Wis1 is not activated by such low concentrations of H<sub>2</sub>O<sub>2</sub> (our unpublished data). Taken together, these data imply that H<sub>2</sub>O<sub>2</sub> can activate Sty1 via multiple mechanisms, and that urmylation regulates Sty1 phosphorylation in a Wis1-independent manner.

Here, it has been shown that urmylation limits the H<sub>2</sub>O<sub>2</sub>-induced activation of Sty1, and that the regulatory mechanism is independent of the upstream MAPK cascade. However, it was unclear whether this effect is mediated by protein modification or tRNA thiolation. To investigate this, H<sub>2</sub>O<sub>2</sub>-induced Sty1 phosphorylation was analysed in *Δurm1* and *Δctu1* cells. While Sty1 phosphorylation was again increased in *Δurm1* cells compared to wild-type cells, Sty1 phosphorylation was slightly *decreased* in *Δctu1* cells relative to wild-type cells (**Fig. 4.10**). This result suggests that the role of Urm1 in post-translational modification of proteins is important for Sty1 regulation. However, the Urm1 substrate(s) controlling Sty1 activation remain unknown, and their identification should be a key objective of any future research.

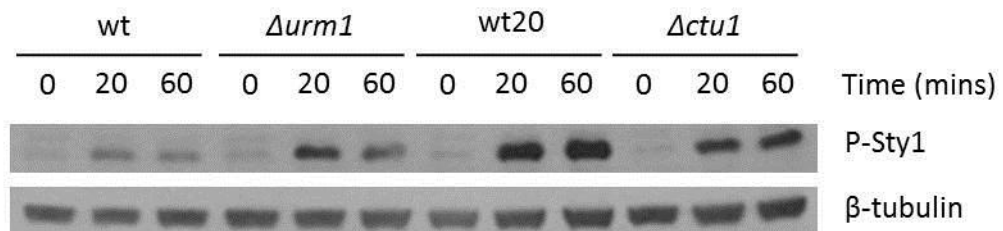
#### 4.2.5. Dysfunctional urmylation also decreases the H<sub>2</sub>O<sub>2</sub>-sensitivity of *Δsty1* cells.

Having established that Urm1 is a negative regulator of Sty1, it was also possible that urmylation has roles in resistance to H<sub>2</sub>O<sub>2</sub> that are independent of Sty1. To investigate



**Figure 4.9. The urmylation pathway regulates Sty1 activation independently of the MAPKK, Wis1.**

Wild-type (wt; CHP429),  $\Delta urm1$  (MF25), Wis1<sup>DD</sup> (MF88) and  $\Delta urm1$  Wis1<sup>DD</sup> (MF85) cells were treated with 1mM H<sub>2</sub>O<sub>2</sub>, and cells harvested at the time-points indicated. Sty1 phosphorylation (P-Sty1) was investigated by western blotting, using anti-phospho-p38 antibodies.  $\beta$ -tubulin levels are shown as a loading control. The above result is representative of two different experiments.



**Figure 4.10. The oxidative stress-induced activation of Sty1 is increased in  $\Delta urm1$  cells, but not in  $\Delta ctu1$  cells.**

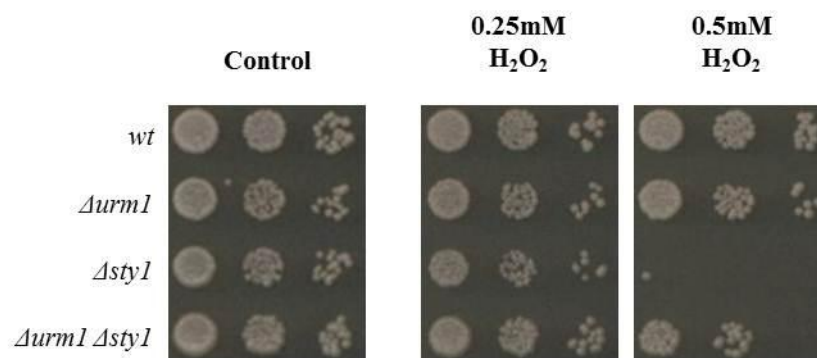
Wild-type (wt; CHP429),  $\Delta urm1$  (MF25), wild-type 20 (DH20) and  $\Delta ctu1$  (DH252) cells were treated with 1mM H<sub>2</sub>O<sub>2</sub> and cells harvested after 0, 20 and 60 minutes. Sty1 phosphorylation (P-Sty1) was investigated by western blotting, using anti-phospho-p38 antibodies.  $\beta$ -tubulin levels are shown as a loading control. This result is representative of three different experiments.

how dysfunctional urmylation affects the oxidative stress sensitivity of *Δsty1* cells, a *ΔurmlΔsty1* strain was constructed by genetic cross between the MF44 and AD22 strains, and H<sub>2</sub>O<sub>2</sub>-sensitivity was investigated. As expected, *Δsty1* cells displayed increased sensitivity to H<sub>2</sub>O<sub>2</sub>, relative to wild-type cells (**Fig. 4.11**), thus emphasising the importance of Sty1 in responses to elevated concentrations of H<sub>2</sub>O<sub>2</sub>. Unexpectedly, *ΔurmlΔsty1* cells were less sensitive to H<sub>2</sub>O<sub>2</sub> than *Δsty1* cells (**Fig. 4.11**). This is reminiscent of the previous result showing that *ΔurmlΔpap1* cells are less sensitive to H<sub>2</sub>O<sub>2</sub> than *Δpap1* cells, and suggests that urmylation has other functions that influence resistance to H<sub>2</sub>O<sub>2</sub>.

The nuclear transport factor CAS has been identified as an Urm1 substrate in mammalian cells (Van der Veen et al., 2011), suggesting that urmylation could influence nuclear import or export in eukaryotes. Significantly, Pap1 cycles in and out of the nucleus before accumulating in the nucleus in response to oxidative stress. If Pap1 nuclear accumulation were increased in *Δurml* cells, it could potentially increase resistance to H<sub>2</sub>O<sub>2</sub> and partially compensate for loss of Sty1 in *ΔurmlΔsty1* cells. Hence, the effect of dysfunctional urmylation on Pap1 localisation was investigated by immunofluorescence. Exposure of cells to 0.2mM H<sub>2</sub>O<sub>2</sub> triggers a rapid accumulation of Pap1 in the nucleus, which is maintained for over 30 minutes before Pap1 begins to relocate to the cytosol (**Fig. 4.12**). There was no obvious difference in the speed or duration of Pap1 nuclear accumulation in *Δurml* cells, relative to wild-type (**Fig. 4.12**), suggesting that dysfunctional urmylation decreases the oxidative stress sensitivity of *Δsty1* cells independently of Pap1.

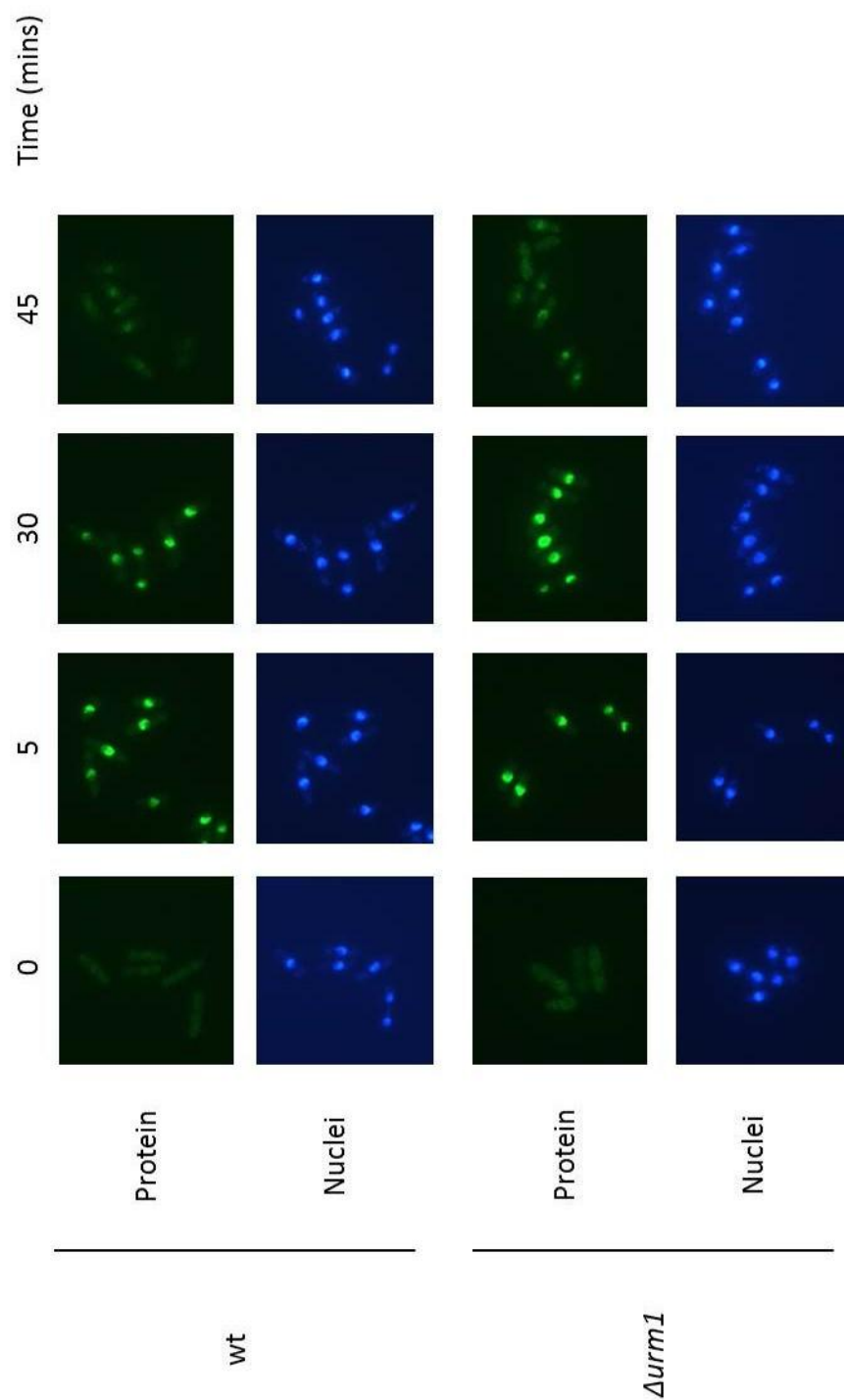
In summary, these data demonstrate that urmylation is important for resistance to oxidative and heat stresses in eukaryotes. In agreement with previous findings in *S. cerevisiae* (Furukawa et al., 2000; Goehring et al., 2003; Leidel et al., 2009), dysfunctional urmylation causes *S. pombe* to become sensitive to diamide as a result of effects on tRNA thiolation, while *Δurml* cells appear to be sensitive to heat due to loss of both tRNA thiolation and protein urmylation. Additionally, urmylation has a role in the regulation of Sty1 activity in *S. pombe*. Urmylation appears to limit H<sub>2</sub>O<sub>2</sub>-induced Sty1 phosphorylation independently of the upstream MAPK cascade, although the underlying molecular mechanism is unknown. It is possible that increased Sty1 activity has implications for resistance to H<sub>2</sub>O<sub>2</sub>, given that dysfunctional urmylation slightly decreases the H<sub>2</sub>O<sub>2</sub>-sensitivity of *Δpap1* cells. However, dysfunctional urmylation also





**Figure 4.11. Loss of the urmylation pathway decreases  $\Delta sty1$  sensitivity to H<sub>2</sub>O<sub>2</sub>.**

Ten-fold serial dilutions of mid-log phase growing wild-type (*wt*; CHP429),  $\Delta urm1$  (MF25),  $\Delta sty1$  (AD22) and  $\Delta urm1 \Delta sty1$  (MF79) cells were spotted onto YE5S plates including the indicated concentrations of H<sub>2</sub>O<sub>2</sub>, and incubated at 30°C for three days.



**Figure 4.12. Dysfunctional urmylation did not cause a detectable effect on the stress-induced nuclear accumulation of Pap1.** To investigate Pap1 localisation, mid-log phase wild-type (wt; CHP429) and *Δurm1* (MF25) cells were treated with 0.2mM H<sub>2</sub>O<sub>2</sub> for the times indicated, and cells were harvested and fixed with 3.7% paraformaldehyde. Pap1 was visualised by indirect immunofluorescence using anti-Pap1 antibodies (Quinn *et al.*, 2002) and Alexa Fluor®-conjugated anti-rabbit antibodies. Nuclei were visualised by DAPI staining. This result is representative of three experiments.

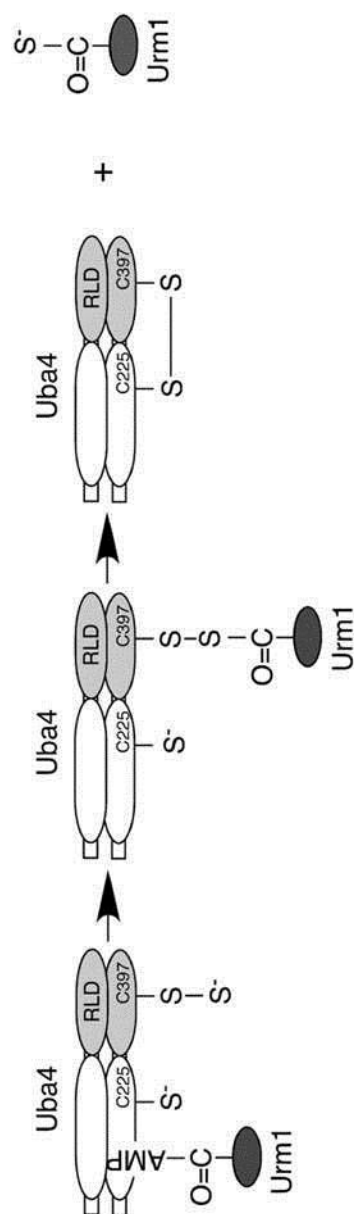
decreases the H<sub>2</sub>O<sub>2</sub>-sensitivity of *Δsty1* cells, suggesting that there are other functions of urmylation that may influence resistance to H<sub>2</sub>O<sub>2</sub> in *S. pombe*.

### **4.3. Oxidation of the Urm1-specific E1, Uba4, and redox regulation of urmylation.**

Previous investigations in *S. cerevisiae* have shown that urmylation is required for resistance to a range of stress conditions (Furukawa et al., 2000; Goehring et al., 2003a). Additionally, urmylation is increased during oxidative stress in both yeast and mammalian cells (Goehring et al., 2003a; Van der Veen et al., 2011), although it remains unclear how oxidative stress increases protein urmylation. Furthermore, the functional consequences of protein urmylation remain unclear, and no conserved Urm1 substrate has yet been identified. The results described in Section 4.2 demonstrate for the first time that urmylation has conserved roles in resistance to oxidative and heat stresses. To expand on these findings, the possible redox regulation of urmylation was investigated in *S. pombe*, and a possible conserved Urm1 substrate was identified.

#### **4.3.1. Pk epitope-tagging of Uba4 at the C-terminus.**

While redox regulation of other ubiquitin/Ubl conjugation pathways is often inhibitory, this is unlikely to be the case for urmylation. Firstly, urmylation is increased during oxidative stress in yeast and mammalian cells (Furukawa et al., 2000; Goehring et al., 2003a). Secondly, no Urm1-specific E2 has yet been identified, making E1-E2 disulfide complex formation unlikely. However, Uba4 may be redox-regulated via the formation of intramolecular disulfide bonds. Indeed, it has been proposed that intramolecular disulfide formation forms part of the catalytic mechanism of Uba4 (**Fig. 4.13**) (Schmitz et al., 2008). In this model, a disulfide bond is formed between Uba4 and Urm1, which is then resolved to an intramolecular disulfide within Uba4, thus releasing



**Figure 4.13. The formation of an intramolecular disulfide bond may be crucial for the catalytic mechanism of Uba4** (Adapted from Schmitz *et al.* 2008, Fig. 8B).

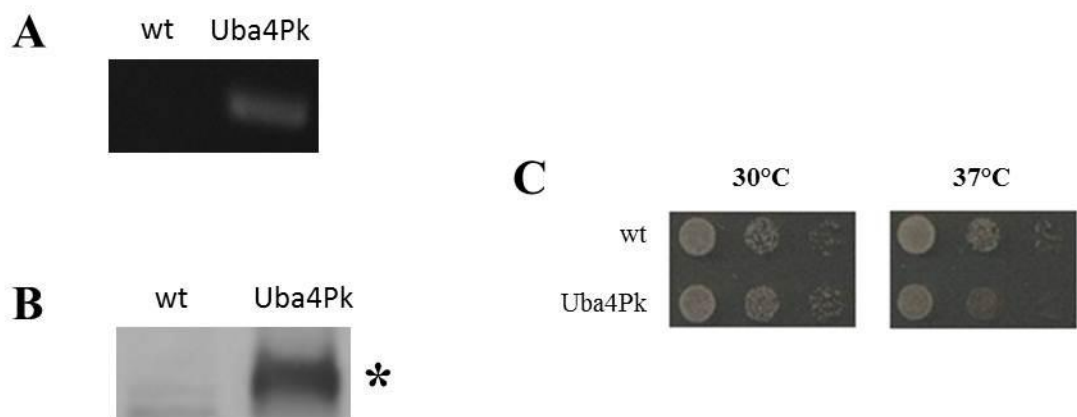
It has been proposed that the adenylation and thiocarboxylation of Urm1 in budding yeast requires the transient formation of a disulfide within the E1, Uba4. In this model, Urm1 forms a thioester bond to a sulphur atom which is itself bonded to a cysteine residue (Cys397) located within the C-terminal RLD of Uba4, as part of a persulfide group. This is resolved by disulfide bond switching, leading to the formation of an intramolecular disulfide between Cys225 and Cys397 of Uba4, and the release of thiocarboxylated Urm1.

thiocarboxylated Urm1. This mechanism requires deprotonation of a non-catalytic cysteine of Uba4, which should make this residue highly-sensitive to oxidation. Therefore, it is possible that cysteine oxidation may regulate the catalytic mechanism of Uba4 in response to oxidative stress. To investigate this possibility, Uba4 was tagged at the C-terminus with three copies of the Pk epitope and expressed from the normal chromosomal locus, using the integration tagging plasmid pRIP42PkC-*uba4* (see Section 2.1.5.1) and the tagging strategy described in **Fig. 3.2**. Successful introduction of pRIP42PkC-*uba4* into the *uba4*<sup>+</sup> locus of CHP429 cells was shown by PCR (**Fig. 4.14A**), and the expression of Uba4Pk was confirmed by western blotting (**Fig. 4.14B**). To determine whether epitope-tagging of Uba4 has any effect on the function of the enzyme, the heat sensitivity of Uba4Pk cells was investigated. While Uba4Pk cells displayed a mild increase in sensitivity to growth at 37°C (**Fig. 4.14C**), *Δurm1* cells were far more sensitive to heat than Uba4Pk cells (**compare Fig. 4.2F and 4.14C**), suggesting that Pk epitope-tagging may slightly affect the activity of Uba4, but the enzyme remains functional.

#### **4.3.2. Uba4 is present in both oxidised and reduced forms, and oxidative stress increases the level of oxidised Uba4.**

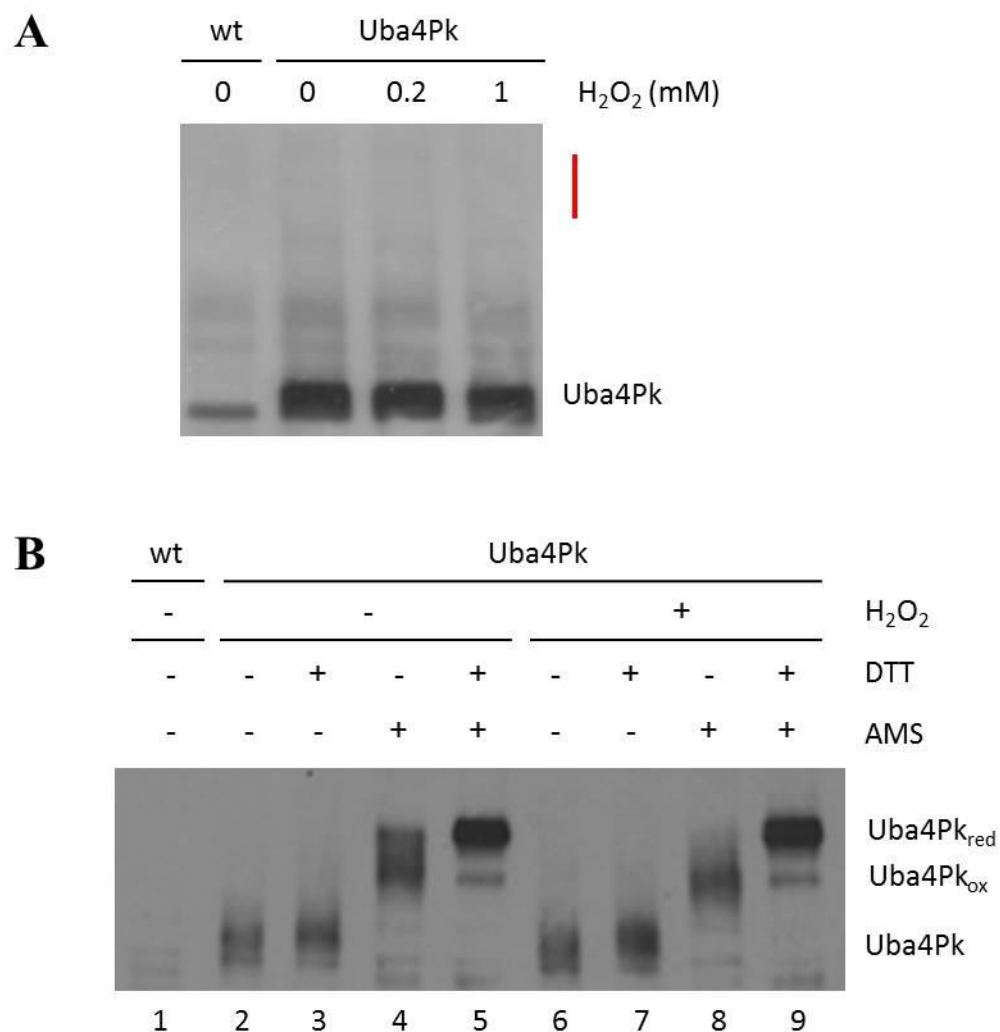
Next, to determine whether Uba4 forms HMW disulfide complexes following cysteine oxidation, Uba4Pk cells were treated with a range of H<sub>2</sub>O<sub>2</sub> concentrations, and cellular extracts were analysed by western blotting. As expected, Uba4 did not appear to form HMW disulfide complexes (**Fig. 4.15A**). Furthermore, there was no evidence of post-translational modification of Uba4 under homeostatic conditions or during oxidative stress, suggesting that any induction of urmylation during oxidative stress does not result from post-translational modification of Uba4.

Although Uba4 does not appear to form HMW disulfide complexes in *S. pombe*, cysteine oxidation may also result in intramolecular disulfide formation. To investigate the possibility that Uba4 includes redox-sensitive cysteines, Uba4Pk cells were exposed to H<sub>2</sub>O<sub>2</sub>, and cellular extracts prepared by acid lysis. The oxidation state of cysteine residues was then investigated by western blot analysis of protein extracts prepared in



**Figure 4.14. Pk epitope-tagging of Uba4 at the C-terminus has no effect on viability and only a minor effect on heat sensitivity.**

**A.** To confirm integration of pRIP42PkC, gDNA was extracted from wild-type (wt; CHP429) cells and from cells transformed with linearised pRIP42PkC-*uba4* to tag Uba4 with Pk epitopes (MF23). Integration of pRIP42PkC-*uba4* at the *uba4<sup>+</sup>* locus was confirmed by PCR using the forward primer Uba4chkf and the reverse primer nmtend (see **Table 2.2**), and gDNA extracts as templates. PCR products were analysed by agarose gel electrophoresis, and a product of ~500bp was taken as confirmation of plasmid integration. **B.** Extracts from wild-type (wt) and Uba4Pk cells were prepared and analysed by western blotting. Pk epitope-tagging of Uba4 was confirmed using anti-Pk antibodies. Uba4Pk (~45kDa, as expected) is indicated by an asterisk. **C.** To investigate whether tagging Uba4 affects the function of the enzyme, ten-fold serial dilutions of mid-log phase wild-type (wt) and Uba4Pk cells were spotted onto solid YE5S media, and incubated at 30°C or 37°C for 3 days.



**Figure 4.15. Uba4 does not form HMW disulfide complexes following H<sub>2</sub>O<sub>2</sub> treatment, but includes redox-sensitive cysteine residues.**

**A.** Uba4Pk (MF23) cells were treated with the indicated concentrations of H<sub>2</sub>O<sub>2</sub> for 20 minutes, and protein extracts prepared by TCA lysis. An extract from wild-type (wt; CHP429) cells expressing untagged protein was used as a control. Protein extracts were analysed by western blotting under non-reducing conditions, using anti-Pk antibodies. The position on the blot including proteins of the M<sub>r</sub> expected for E1-E2 complexes is indicated by a red line. This result is representative of three different experiments. **B.** Uba4Pk cells were incubated with or without H<sub>2</sub>O<sub>2</sub> for 20 minutes, and cellular extracts were prepared by TCA lysis. During preparation, samples were treated with the cysteine-reducing agent DTT and/or the cysteine-alkylating agent AMS, as indicated. An extract from wild-type (wt) cells was also prepared, as a control. The oxidation state of Uba4 was analysed by western blotting, using anti-Pk antibodies. The oxidised (Uba4<sub>ox</sub>) and reduced (Uba4<sub>red</sub>) forms of Uba4 are indicated. This result is representative of three different experiments.

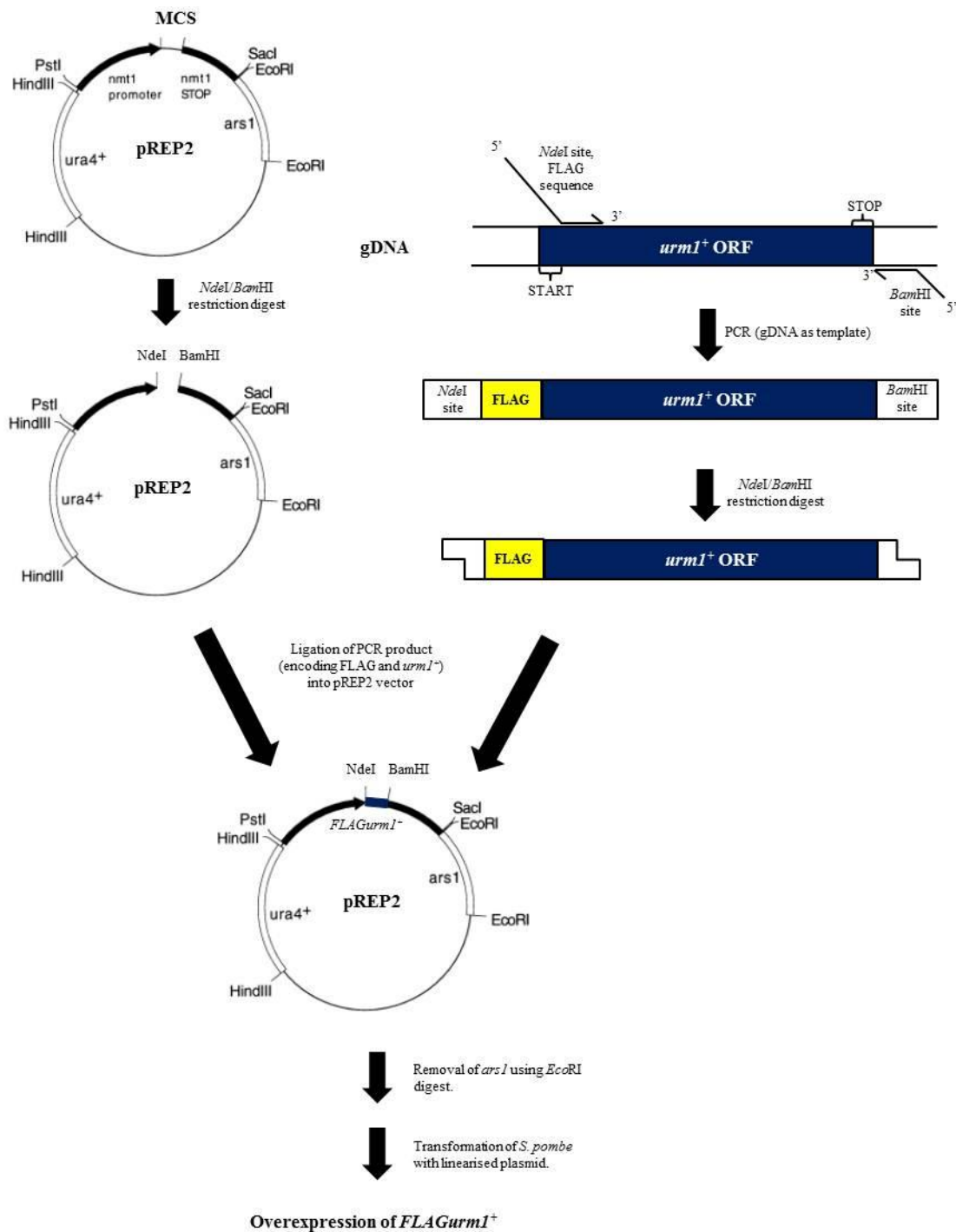
the presence or absence of the reducing agent DTT and the alkylating agent AMS. Uba4 includes 15 cysteine residues, and while the majority of these cysteines appear to be reduced under homeostatic conditions, a significant proportion of Uba4 is oxidised (Uba4<sub>ox</sub>) (**Fig. 4.15B, lane 4**). Furthermore, far more Uba4<sub>ox</sub> was detected following H<sub>2</sub>O<sub>2</sub> treatment (**Fig. 4.15B, compare lanes 4 and 8**), strongly suggesting that Uba4 includes redox-sensitive cysteine residues.

The observation that Uba4 includes redox-sensitive cysteines and becomes more oxidised following H<sub>2</sub>O<sub>2</sub> treatment is consistent with redox regulation of the enzyme. Oxidation of Uba4 might be expected to increase the activity of the enzyme and promote protein urmylation, given that urmylation is induced by oxidative stress in other organisms (Goehring et al., 2003a; Van der Veen et al., 2011). However, it is unclear which cysteines are oxidised by H<sub>2</sub>O<sub>2</sub>, or how cysteine oxidation affects Uba4 function. As described above, the catalytic mechanism of Uba4 is very different from those of other E1s in eukaryotes, and may involve intramolecular disulfide formation (**Fig. 4.13**). Hence, it is possible that the redox-sensitive cysteine residues are those involved in the activation and thiocarboxylation of Urm1. How Uba4 oxidation and intramolecular disulfide formation affects Urm1 conjugation to proteins and sulphur transfer to tRNAs will require further investigation.

#### **4.3.3. Investigation of the effects of oxidative stress on protein urmylation.**

Although protein urmylation is increased during oxidative stress in other organisms, this had not been investigated in *S. pombe*. Previous investigations in *S. cerevisiae* have used FLAG epitope-tagged Urm1 to investigate the effect of oxidative stress on protein urmylation (Goehring et al., 2003a). Hence, the expression plasmid pREP2-FLAG<sub>Urm1</sub> was constructed (see Section 2.1.5.2) to express Urm1 tagged at the N-terminus with FLAG using the thiamine-repressible *nmt* promoter, using the strategy shown in **Fig. 4.16** (Craven et al., 1998). This expression plasmid was linearised and introduced into CHP429 cells, allowing homologous recombination into the *urm1*<sup>+</sup> locus. The expression of FLAGUrm1 and the presence of FLAGUrm1 conjugates in cellular extracts were then investigated by western blotting. Previous studies in *S. cerevisiae*





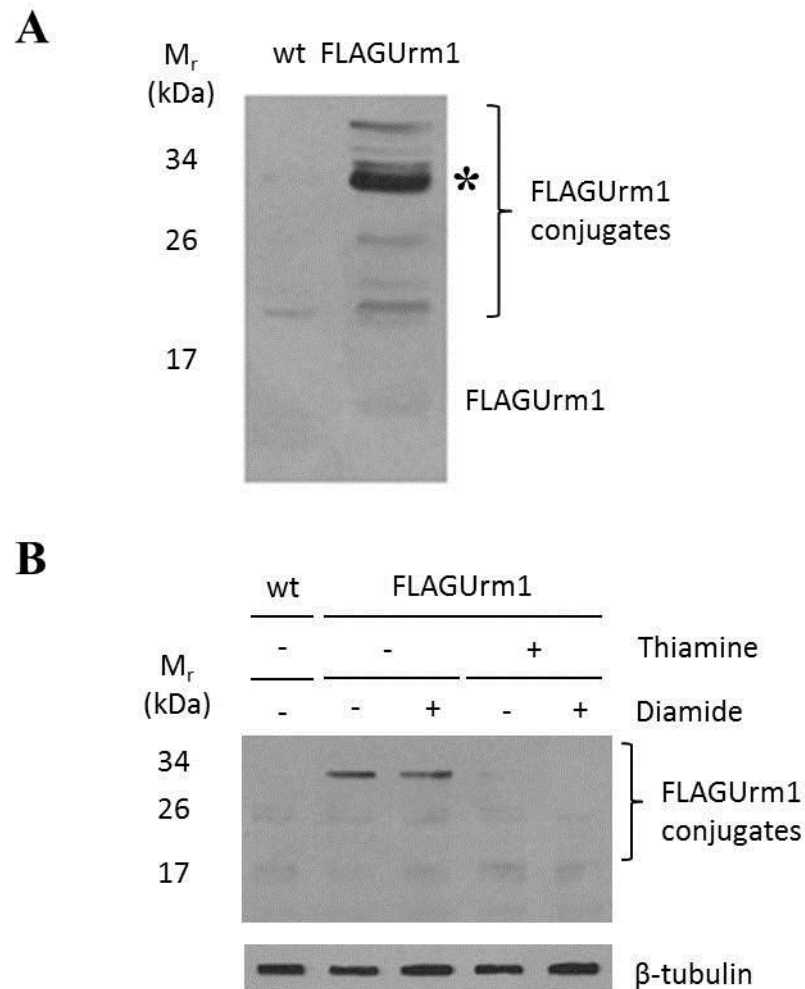
**Figure 4.16. Strategy used to express FLAG-tagged Urm1 by introducing recombinant pREP2 into *S. pombe*** (Adapted from Craven *et al.* 1998, Fig. 5).

The *urm1<sup>+</sup>* gene was amplified from a wild-type (CHP429) gDNA template by PCR using the FLAGUrm1oef and FLAGUrm1oer oligonucleotide primers (see Table 2.2), which introduced NdeI and BamHI restriction sites before the START codon and after the STOP codon, respectively. The forward primer also contains the DNA sequence encoding the FLAG epitope (5'-GATTACAAGGACGATGACGACAAG-3'). After digestion with NdeI and BamHI restriction enzymes, the PCR product was ligated with NdeI/BamHI-digested pREP2 vector. The *ars1* sequence was removed from the plasmid by digestion with EcoRI. To express FLAG epitope-tagged Urm1, pREP2-FLAGUrm1 was linearised and introduced into *S. pombe*, and positive transformants were selected by growth on media lacking uracil.

have shown that NEM must be included in the lysis buffer to retain Urm1 conjugates during extraction (Goehring et al., 2003a). Although the reason for this is unclear, it suggests that an unknown ULP can remove Urm1 from substrates. Hence, extracts were prepared with NEM included in the lysis buffer. Although low levels of unconjugated FLAGUrm1 were detected, the majority of FLAGUrm1 appears to be conjugated to proteins under homeostatic conditions (**Fig. 4.17A**). Many proteins appear to be urmylated in fission yeast, as in other organisms, and a band of ~30kDa was particularly abundant. This FLAGUrm1 conjugate was named Urs30 (Urm1 substrate, 30kDa) (**Fig. 4.17A**).

Having established that several proteins are urmylated in *S. pombe*, the effect of oxidative stress on urmylation was investigated. While urmylation is induced by many oxidative stress agents in other organisms, the effect is most significant when cells are treated with diamide (Goehring et al., 2003a; Van der Veen et al., 2011). Therefore, FLAGUrm1 cells were treated with diamide, and cellular extracts prepared and analysed by western blotting. Despite the inclusion of NEM in the lysis buffer, the detection of urmylated proteins was variable. Indeed, Urs30 was the only protein consistently detected in cellular extracts (**Fig. 4.17B** and data not shown). Importantly, Urs30 could not be detected in extracts from cells grown in media containing thiamine, confirming that this band is a FLAGUrm1 conjugate. However, Urs30 levels were unaffected following exposure to diamide (**Fig. 4.17A**), or following exposure to H<sub>2</sub>O<sub>2</sub> (data not shown). Thus, the urmylation of at least one protein was unaffected by oxidative stress in *S. pombe*. Despite this, it remains possible that oxidative stress increases protein urmylation in *S. pombe*, and that a more robust method of detection is required to show this. Moreover, Urs30 could be maximally urmylated under normal conditions, meaning that increasing overall urmylation would not increase levels of Urs30. In the future, it would be interesting to investigate how oxidative stress affects the urmylation of other proteins in *S. pombe*, and relate this to redox regulation of Uba4.

#### **4.3.4. CAS/Xpo2 may be a conserved Urm1 substrate in eukaryotes.**

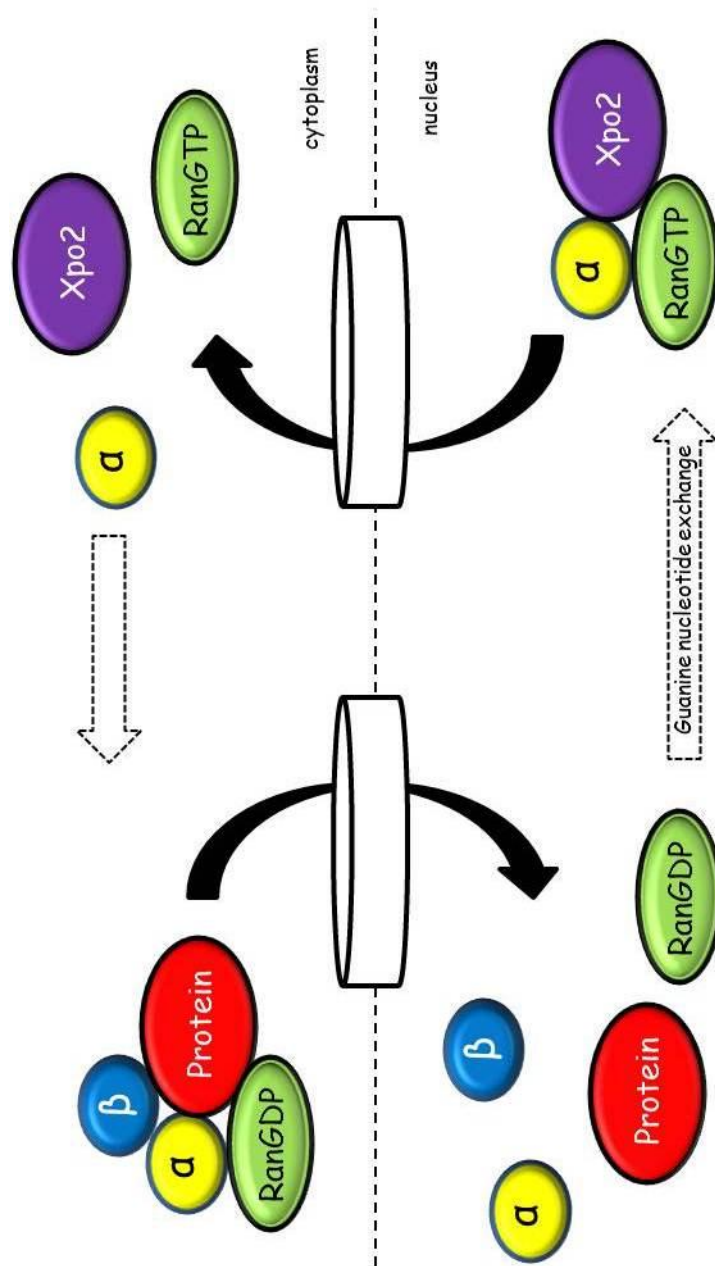


**Figure 4.17. Expression of FLAGUrm1 from the chromosomal locus allows the detection of urmylated proteins in *S. pombe*.**

**A.** Extracts from FLAGUrm1-expressing (MF83) and wild-type (wt; CHP429) cells were analysed by western blotting, using anti-FLAG antibodies. Predicted FLAGUrm1 and FLAGUrm1 conjugates are indicated. Urs30 is indicated by an asterisk. **B.** FLAGUrm1-expressing and wild-type (wt) cells were grown in the presence or absence of 5 µg/ml thiamine, and/or treated with 4mM diamide for 20 minutes, as indicated. Cell extracts were prepared, and urmylated proteins identified by western blotting using anti-FLAG antibodies. Levels of β-tubulin are shown as a loading control.

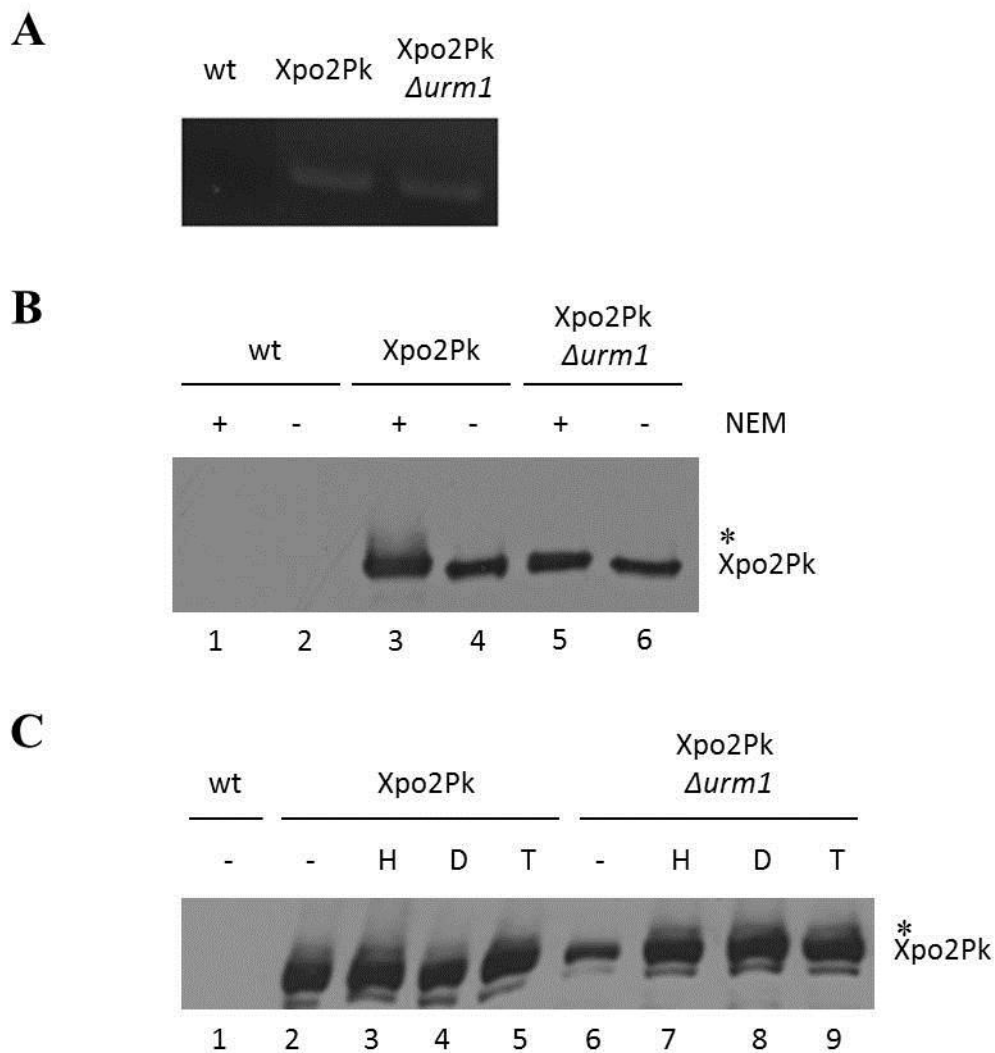
Urmylation has been linked to oxidative stress responses in yeast and mammals, and research in *S. cerevisiae* suggests that sulphur transfer to tRNAs is important for resistance to oxidative stress (Leidel et al., 2009). However, the induction of protein urmylation during oxidative stress suggests that this is also important for survival, and the urmylation of specific, conserved proteins could be important for stress resistance in eukaryotes. Interestingly, the nuclear transport protein CAS (also known as exportin-2) has recently been identified as an Urm1 substrate in mammals (Van der Veen et al., 2011). CAS mediates the nuclear export of the importin- $\alpha$  protein, which is itself required for the nuclear import of proteins containing a nuclear localisation sequence (NLS) (**Fig. 4.18**) (Kunzler and Hurt, 1998; Kutay et al., 1997; Solsbacher et al., 1998). How urmylation of CAS affects the function of the protein remains unknown. However, given that the nuclear import of proteins such as Pap1 and Sty1 is key for oxidative stress response mechanisms in *S. pombe* (see Section 1.3), it is feasible that the urmylation of fission yeast CAS homologues could influence stress resistance. Indeed, Pap1 nuclear import is mediated by the importin- $\alpha$  homologues Imp1 and Cut15 (Umeda et al., 2005). The fission yeast CAS homologue is encoded by a locus on chromosome II (SPBC30B4.05), has a predicted  $M_r$  of ~110kDa, and has previously been identified as a nuclear transport factor similar to the budding yeast CAS homologue, Cse1 (Chen et al., 2004). The fission yeast CAS homologue will be named Xpo2 for the purposes of this research.

First, to determine whether Xpo2 is urmylated, the integration tagging plasmid pRIP42PkC-*xpo2* was constructed (see Section 2.1.5.1) using the strategy outlined in **Fig. 3.2**, and introduced into wild-type (CHP429) and *Δurm1* (MF25) strains to express Pk epitope-tagged Xpo2 from the normal chromosomal locus. Integration of pRIP42PkC-*xpo2* at the *xpo2*<sup>+</sup> locus was confirmed by PCR (**Fig. 4.19A**). To confirm the expression of Xpo2Pk, and investigate its possible urmylation, extracts were prepared from wild-type and *Δurm1* cells expressing Xpo2Pk, and analysed by western blotting. Xpo2Pk has a  $M_r$  of ~110kDa, as expected (**Fig. 4.19B**). Excitingly, a slower mobility form of Xpo2Pk was detected when NEM was included in the lysis buffer, but not in the absence of NEM (**Fig. 4.19B, compare lanes 3 and 4**). This apparent post-translational modification of Xpo2Pk causes a  $M_r$  increase of ~10kDa, which is consistent with ubiquitin/Ubl conjugation. Furthermore, this slower mobility form of Xpo2Pk is absent from cell extracts prepared from *Δurm1* cells (**Fig. 4.19B, compare lanes 3 and 5**). It is possible that Xpo2 is not directly modified by Urm1, and that



**Figure 4.18. CAS/Xpo2 facilitates nuclear import by recycling importin- $\alpha$ .**

The nuclear import of many proteins with a nuclear localisation signal (NLS) requires the formation of a nuclear import complex. As well as the target protein, these complexes include the small GTPase Ran (bound to GDP), and importins  $\alpha$  and  $\beta$ . This complex associates with the nuclear pore complex (NPC), allowing translocation into the nucleus. The target protein is then released into the nucleus. To allow nuclear import to continue, importin- $\alpha$  is exported from the nucleus as part of a complex involving CAS/Xpo2 and Ran (bound to GTP), again via the NPC. Importin- $\alpha$  can then form further nuclear import complexes (Kunzler and Hurt, 1998; Kutay *et al.*, 1997; Solsbacher *et al.*, 1998).



**Figure 4.19. The CAS homologue, Xpo2, appears to be urmylated in *S. pombe*.**

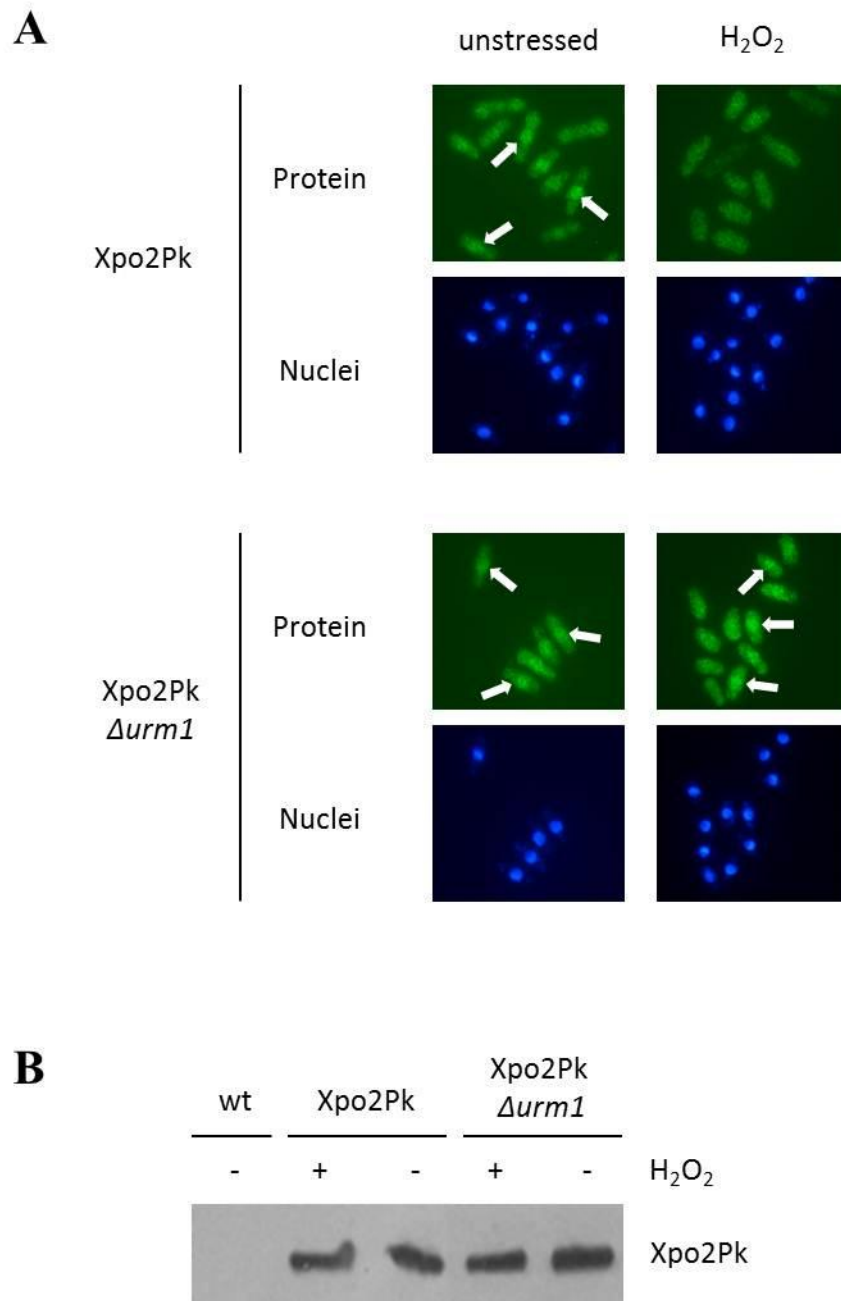
**A.** To confirm integration of pRIP42PkC, yeast gDNA was extracted from wild-type (wt; CHP429) cells and from wild-type and  $\Delta urm1$  cells transformed with linearised, recombinant pRIP42PkC to tag Xpo2 with Pk epitopes (MF47 and MF49, respectively). Integration of pRIP42PkC-*xpo2* at the *xpo2*<sup>+</sup> locus was confirmed by PCR using the forward primer Xpo2chkf, which binds to a site within the ORF, and the reverse primer nmtend (see Table 2.2), and gDNA extracts as templates. PCR products were analysed by agarose gel electrophoresis, and products of ~600bp confirmed plasmid integration. **B.** Protein extracts were prepared from Xpo2Pk (MF47), Xpo2Pk  $\Delta urm1$  (MF49), and wild-type (wt; CHP429) cells using lysis buffer containing 20mM NEM, or standard lysis buffer, as indicated. Extracts were analysed by western blotting, using anti-Pk antibodies. The putative Xpo2Pk-Urm1 conjugate is indicated by an asterisk. This experiment was repeated twice, giving the same result each time. **C.** Xpo2Pk and Xpo2Pk  $\Delta urm1$  cells were treated with 1mM H<sub>2</sub>O<sub>2</sub> (H), 2mM diamide (D), or 0.5mM *t*-BOOH (T) for 20 minutes, as indicated. Cells were harvested, extracts prepared using lysis buffer containing 20mM NEM, and protein analysed by western blotting using anti-Pk antibodies. Extracts from wild-type (wt) cells were also prepared and analysed. Modified forms of Xpo2Pk are indicated by an asterisk. This experiment was repeated and gave the same result.

urmylation somehow controls the post-translational modification of Xpo2 by another protein. Nevertheless, this result strongly suggests that Xpo2 is urmylated and, taken together with findings in mammalian cells, suggests that CAS/Xpo2 is the first conserved Urm1 substrate to be identified in eukaryotes.

Having established that post-translational modification of Xpo2 is influenced by Urm1, it was possible that Xpo2 modification is increased during oxidative stress. Xpo2Pk and Xpo2Pk *Δurm1* cells were treated with H<sub>2</sub>O<sub>2</sub>, diamide or *t*-BOOH, and protein extracts were prepared in the presence of NEM. Again, a modified form of the protein was detected in extracts from Xpo2Pk cells, but not in extracts from Xpo2Pk *Δurm1* cells (**Fig. 4.19C, compare lanes 2 and 6**). Notably, modification of Xpo2Pk was not increased when cells were treated with H<sub>2</sub>O<sub>2</sub>, diamide or *t*-BOOH (**Fig. 4.19C, lanes 2-5**). However, this interpretation is complicated by the appearance of slower mobility forms of Xpo2Pk in a *Δurm1* background, following treatment of cells with H<sub>2</sub>O<sub>2</sub>, diamide or *t*-BOOH (**Fig. 4.19C, lanes 6-9**). The nature of this modification is unknown, but the resulting M<sub>r</sub> shift is again consistent with ubiquitin/Ubl conjugation. It is possible that Xpo2 is modified by a ubiquitin/Ubl other than Urm1 during oxidative stress.

Although urmylated proteins have been identified in both yeast and mammals, no functional consequence of urmylation has yet been shown. As described previously, CAS/Xpo2 cycles between the nucleus to the cytoplasm to 'recycle' importin- $\alpha$ , allowing the nuclear import of proteins to continue. Hence, it was possible that urmylation influences the localisation of Xpo2. To investigate this possibility, Xpo2Pk localisation was analysed by indirect immunofluorescence. Under homeostatic conditions, Xpo2Pk was associated with the nucleus (**Fig. 4.20A, unstressed**). This finding is consistent with a previous report showing that Xpo2 localises to the nuclear rim (Chen et al., 2004). Interestingly, when cells were treated with 1mM H<sub>2</sub>O<sub>2</sub>, Xpo2Pk appeared to disperse into the cytoplasm. In contrast, Xpo2Pk remained associated with the nucleus following H<sub>2</sub>O<sub>2</sub> treatment in *Δurm1* cells (**Fig. 4.20A, H<sub>2</sub>O<sub>2</sub>**). Furthermore, Xpo2Pk levels were not affected by H<sub>2</sub>O<sub>2</sub> in wild-type or *Δurm1* cells (**Fig. 4.20B**). These results suggest that urmylation has an important role in controlling CAS/Xpo2 localisation during oxidative stress. During oxidative stress in mammalian cells, nuclear import is largely prevented due to the collapse of the Ran gradient across the nuclear membrane (Miyamoto et al., 2004). This may affect the nuclear import and export of





**Figure 4.20. Dysfunctional urmylation causes Xpo2 to be retained in the nucleus during oxidative stress.**

**A.** Xpo2Pk (MF47) and Xpo2Pk  $\Delta$ urm1 (MF49) cells were fixed with 3.7% paraformaldehyde before and after 60 minutes' exposure to 1mM H<sub>2</sub>O<sub>2</sub>, as indicated. Xpo2Pk was visualised by indirect immunofluorescence using mouse anti-Pk antibodies and Alexa Fluor®-conjugated anti-mouse antibodies. Nuclei were visualised by DAPI staining. Nuclear Xpo2 is indicated by arrows. The above result is representative of three experiments. **B.** Xpo2Pk and Xpo2Pk  $\Delta$ urm1 cells were incubated with or without 1mM H<sub>2</sub>O<sub>2</sub> for 60 minutes, as indicated. An extract from wild-type (wt; CHP429) cells expressing untagged protein was used as a control. Cellular extracts were prepared under reducing conditions, and levels of Xpo2 were examined by western blotting, using anti-Pk antibodies. This experiment was repeated and gave the same result.



proteins during stress. It is possible that post-translational modification of Xpo2 controls the function of the protein under homeostatic conditions, and may also be important for the control of nuclear trafficking during oxidative stress. Clearly, the functional consequences of Urm1 conjugation to CAS/Xpo2 require further investigation.

#### 4.4. Discussion

Urm1 is a conserved Ubl conjugation pathway in eukaryotes, with functions in both protein modification and sulphur transfer to tRNAs. Studies in budding yeast have shown that the urmylation pathway is important for resistance to numerous stress conditions, including oxidative stress and elevated temperature (Furukawa et al., 2000; Goehring et al., 2003a). However, it is unclear why urmylation is important for stress resistance. Additionally, although protein urmylation is increased during oxidative stress in yeast and mammalian cells (Goehring et al., 2003a; Van der Veen et al., 2011), how this is achieved remains unknown. Furthermore, urmylation has not previously been investigated in fission yeast. In this chapter, links between urmylation and oxidative stress responses were investigated in *S. pombe*. It appears that urmylation has conserved roles in resistance to oxidative and heat stresses in eukaryotes. Furthermore, Urm1 appears to regulate the H<sub>2</sub>O<sub>2</sub>-induced activation of the SAPK, Sty1, and dysfunctional urmylation decreases the sensitivity of *Δsty1* and *Δpap1* cells to H<sub>2</sub>O<sub>2</sub>. Additionally, the Urm1-specific E1, Uba4, contains redox-sensitive cysteine residues, and CAS/Xpo2 may be a conserved Urm1 substrate in eukaryotes.

Fission yeast *Δurm1* cells display increased sensitivity to heat and to oxidative stress agents such as diamide. This is also the case for budding yeast cells lacking a functional urmylation pathway (Furukawa et al., 2000; Goehring et al., 2003a). By comparing the stress sensitivities of *Δurm1* cells with those of strains that are defective in tRNA thiolation, it can be concluded that *Δurm1* cells are sensitive to diamide due to loss of sulphur transfer to tRNAs, and sensitive to heat due to loss of both sulphur transfer and

protein urmylation. Interestingly, studies in *S. cerevisiae* have shown that tRNA overexpression rescues the diamide-sensitivity of *Urm1* and *Uba4* cells (Leidel et al., 2009), indicating that tRNA modification is also important for resistance to diamide in budding yeast. However, how tRNA overexpression affects the heat sensitivity of *Urm1* and *Uba4* cells was not shown (Leidel et al., 2009). This is a striking omission, given that heat sensitivity is perhaps the best-known phenotype of budding yeast cells lacking an urmylation pathway. It is possible that tRNA overexpression did not fully rescue heat sensitivity in *S. cerevisiae*. Taken together, these results indicate that the role of Urm1 in tRNA thiolation is important for resistance to oxidative stress in eukaryotes, while both tRNA thiolation and protein urmylation may be important for resistance to heat. If this is the case, it would be interesting to identify Urm1 substrates that are important for growth at elevated temperatures.

It is perhaps surprising that protein urmylation does not appear to be important for resistance to diamide, given that diamide strongly induces urmylation in *S. cerevisiae* and human cells (Goehring et al., 2003b; Van der Veen et al., 2011). Although it is unclear how urmylation is induced during oxidative stress, fission yeast Uba4 appears to include redox-sensitive cysteines within its structure. How redox regulation of Uba4 affects the function of the enzyme is unclear. Given that urmylation is increased during oxidative stress in yeast and mammals (Goehring et al., 2003a; Van der Veen et al., 2011), it is possible that cysteine oxidation increases the activity of Uba4. However, no increase in protein urmylation was detected during oxidative stress in *S. pombe*, although it remains possible that this was due to technical issues, and the effect of oxidative stress on urmylation could be investigated more thoroughly by refining the technique used to analyse protein urmylation.

A single urmylated protein was consistently detected in cell extracts, and has been named Urs30. In *S. cerevisiae*, the most abundant urmylated protein is Ahp1 (Goehring et al., 2003a), and the Ahp1-Urm1 conjugate has a  $M_r$  of ~30kDa. Interestingly, this is roughly the same  $M_r$  as Urs30. It is tempting to speculate that the *S. pombe* homologue of Ahp1, Pmp20, is also urmylated, and that Urs30 is actually a Pmp20-Urm1 conjugate. In support of this hypothesis, previous investigations in our lab showed that a large pool of cellular Pmp20 is post-translationally modified, leading to a  $M_r$  increase of ~10kDa (V. Findlay, PhD Thesis, Newcastle University), which is consistent with ubiquitin/Ubl conjugation. The possible urmylation of Pmp20 could be investigated

using mass spectrometry, and the effect of urmylation on the function of Pmp20 could also be investigated. In a recent report, it was proposed that urmylation of Ahp1 may inhibit its reactivation by Trx (Lian et al., 2012). Furthermore, urmylation in *S. cerevisiae* is decreased when cells are exposed to *t*-BOOH (Goehring et al., 2003a). Thus, decreased urmylation may be beneficial when *S. cerevisiae* are exposed to *t*-BOOH, as Ahp1 activity would be increased, thus allowing the enzyme to remove *t*-BOOH from the cell. However, *S. cerevisiae*  $\Delta$ *urm1* cells are not sensitive to *t*-BOOH, making this unlikely (Goehring et al., 2003a). If Pmp20 is indeed an Urm1 substrate, it would be interesting to investigate how urmylation of Pmp20 affects the activity of the enzyme, and whether this is important for oxidative stress resistance.

As well as the possible urmylation of Pmp20, Xpo2 could be an Urm1 substrate in *S. pombe*. Mass spectrometry may be required, to confirm that the modified form of Xpo2 that is absent in  $\Delta$ *urm1* cells is an Xpo2-Urm1 conjugate. Significantly, the Xpo2 homologue CAS is urmylated in mammalian cells (Van der Veen et al., 2011). Hence, CAS/Xpo2 could be the first example of a conserved Urm1 substrate in eukaryotes. Furthermore, urmylation of Xpo2 could influence the localisation of the protein. Treatment with H<sub>2</sub>O<sub>2</sub> causes Xpo2 to gradually disperse into the cytosol in wild-type cells. However, this dispersal is much slower in  $\Delta$ *urm1* cells. This could be highly significant, as no molecular consequence of protein urmylation has previously been reported. However, it is unclear how urmylation of Xpo2 may affect nuclear transport as a whole. In eukaryotes, CAS/Xpo2 facilitates the nuclear export of importin- $\alpha$ , which is necessary to allow nuclear import to continue. Therefore, any change in Xpo2 localisation might affect the nuclear import of other proteins, although the H<sub>2</sub>O<sub>2</sub>-induced nuclear accumulation of Sty1 and Pap1 did not appear to be affected in  $\Delta$ *urm1* cells. Clearly, the possible urmylation of Xpo2, and how this may affect the function of Xpo2, warrants further investigation.

Although dysfunctional urmylation does not cause *S. pombe* cells to become sensitive to H<sub>2</sub>O<sub>2</sub>, the H<sub>2</sub>O<sub>2</sub>-induced activation of Sty1 is increased and prolonged in  $\Delta$ *urm1* cells, relative to wild-type. How and why Sty1 activation is increased in  $\Delta$ *urm1* cells remains unclear. However, it is possible that Urm1 somehow regulates Sty1 activation independently of the upstream MAPKK Wis1, as H<sub>2</sub>O<sub>2</sub> treatment increased Sty1 phosphorylation in cells expressing a constitutively-active form of Wis1. This contradicts previous reports that Sty1 activation by H<sub>2</sub>O<sub>2</sub> is entirely dependent on the

upstream MAPK cascade (Nguyen and Shiozaki, 1999; Shiozaki et al., 1998). However, during these previous investigations, Sty1 phosphorylation was examined using an antibody raised against epitope-tagged Sty1, whereas Sty1 phosphorylation was investigated here by using an antibody raised against wild-type Sty1. Hence, it is likely that H<sub>2</sub>O<sub>2</sub> activates Sty1 independently of Wis1. In support of this, Sty1 activation by cadmium may involve ROS-dependent inhibition of Pyp1 (Zhou et al., 2010), possibly representing a mechanism by which H<sub>2</sub>O<sub>2</sub> can activate Sty1 independently of Wis1. In contrast, Sty1 activation by osmotic stress is entirely dependent on the MAPK cascade (Zhou et al., 2010), and Sty1 phosphorylation by osmotic stress was increased equally in wild-type and *Urm1* cells. Taken together, these findings support the hypothesis that urmylation limits Sty1 activation by regulating a Wis1-independent mechanism of Sty1 phosphorylation.

The mechanism by which Urm1 regulates Sty1 phosphorylation is unknown. One possibility is that Urm1 regulates Sty1 via the TOR pathway. In *S. cerevisiae*, Urm1 has an undefined role in regulating signalling by Tor2 (Rubio-Teixeira, 2007), and overexpression of a rapamycin-resistant *TOR2* allele rescues the rapamycin-sensitivity of *Urm1* cells (Goehring et al., 2003), indicating that it is important to maintain TOR signalling in *Urm1* cells. If urmylation is also involved in regulating TOR signalling in *S. pombe*, this could have implications for Sty1 activity, as the fission yeast Tor2 homologue, Tor1, inhibits the eIF2 $\alpha$  kinase Gcn2, resulting in stabilisation of *pyp2*<sup>+</sup> mRNA and dephosphorylation of Sty1 (Petersen and Nurse, 2007). However, when Tor1 activity is decreased, Pyp2 levels are decreased, thus preventing dephosphorylation of Sty1. Therefore, if Urm1 promotes Tor1 signalling, loss of Urm1 could also cause Pyp2 levels to decrease, resulting in increased Sty1 phosphorylation. This model is represented in **Fig. 4.21**. Interestingly, Sty1 promotes translation during stress by preventing eIF2 $\alpha$  phosphorylation by Gcn2 (Dunand-Sauthier et al., 2005), and Sty1 is known to interact with translation factors (Asp et al., 2008). Thus, TOR, Urm1 and Sty1 may work together to control the rate of translation and translational fidelity, which may be important for stress resistance.

It is possible that increased Sty1 phosphorylation in *Urm1* cells has implications for oxidative stress resistance. Given that *Urm1* $\Delta$ *pap1* cells are less sensitive to H<sub>2</sub>O<sub>2</sub> than *pap1* cells, increased Sty1 phosphorylation may partially compensate for loss of Pap1. It would be interesting to investigate Atf1-dependent gene expression in *Urm1* cells, as

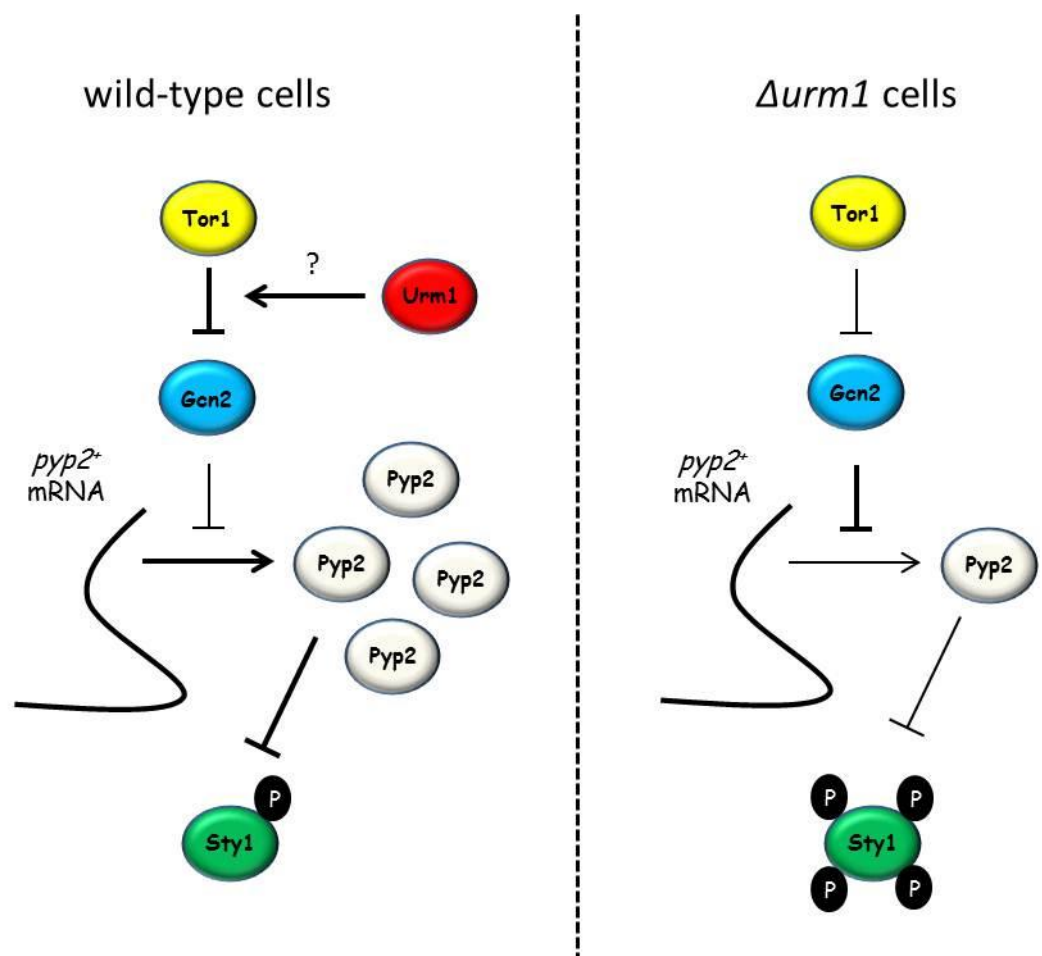


Figure 4.21. A possible model for increased Sty1 activation in  $\Delta urm1$  cells.

increased Sty1 activity could increase antioxidant gene expression via Atf1. While this remains a possibility, it is curious that *Δurm1Δsty1* cells are less sensitive to H<sub>2</sub>O<sub>2</sub> than *Δsty1* cells. It is possible that in addition to Sty1, Urm1 also limits the stress-induced activation of a second SAPK, Pmk1, thus allowing Pmk1 to mediate a compensatory oxidative stress response in *Δurm1Δsty1* cells. Indeed, urmylation may regulate the activity of phosphatases such as Ptc1 or Ptc3, which can target both Sty1 and Pmk1 (Nguyen and Shiozaki, 1999; Takada et al., 2007). Thus, the phosphorylation of both Sty1 and Pmk1 would be increased in *Δurm1* cells, relative to wild-type. However, Pmk1 phosphorylation could not be analysed to test this hypothesis. The mechanism by which Urm1 regulates Sty1 phosphorylation requires further characterisation, and may improve understanding of SAPK regulation in *S. pombe*. Moreover, it is possible that urmylation can regulate the activity of SAPKs in other organisms, given that the urmylation pathway and SAPK cascades are both highly-conserved in eukaryotes.

Finally, Atf1 protein levels were increased in *Δurm1* cells relative to wild-type. This suggests that urmylation may regulate Atf1 levels. Sty1 stabilises *atf1*<sup>+</sup> mRNA both in stressed and unstressed conditions (Shiozaki and Russell, 1996), but basal Sty1 phosphorylation was not increased in *Δurm1* cells relative to wild-type, ruling out the possibility that Atf1 levels are increased in *Δurm1* cells due to increased Sty1 activity. However, there are other possible explanations. Upf1 is a RNA helicase which promotes the stabilisation of *atf1*<sup>+</sup> mRNA (Rodriguez-Gabriel et al., 2006). Interestingly, the mammalian homologue of Upf1 was recently identified as an urmylation substrate (Van der Veen et al., 2011). It is possible that Upf1 is also an urmylation substrate, and that urmylation of Upf1 inhibits its role in mRNA stabilisation. Thus, in *Δurm1* cells, the stability of *atf1*<sup>+</sup> mRNA may be increased, resulting in increased Atf1 protein synthesis. This hypothesis needs to be tested, and Atf1-dependent gene expression in *Δurm1* cells requires investigation. However, if it were true, it would represent another link between urmylation and oxidative stress responses in *S. pombe*.



## Extra References

- Ashraf, S.S., Ansari, G., Guenther, R., Sochacka, E., Malkiewicz, A., and Agris, P.F. (1999). The uridine in "U-turn": contributions to tRNA-ribosomal binding. *RNA* 5, 503-511.
- Chen, D., Wilkinson, C.R., Watt, S., Penkett, C.J., Toone, W.M., Jones, N., and Bahler, J. (2008). Multiple pathways differentially regulate global oxidative stress responses in fission yeast. *Molecular biology of the cell* 19, 308-317.
- Chen, X.Q., Du, X., Liu, J., Balasubramanian, M.K., and Balasundaram, D. (2004). Identification of genes encoding putative nucleoporins and transport factors in the fission yeast *Schizosaccharomyces pombe*: a deletion analysis. *Yeast* 21, 495-509.
- Craven, R.A., Griffiths, D.J., Sheldrick, K.S., Randall, R.E., Hagan, I.M., and Carr, A.M. (1998). Vectors for the expression of tagged proteins in *Schizosaccharomyces pombe*. *Gene* 221, 59-68.
- Dewez, M., Bauer, F., Dieu, M., Raes, M., Vandenhaute, J., and Hermand, D. (2008). The conserved Wobble uridine tRNA thiolase Ctu1-Ctu2 is required to maintain genome integrity. *Proceedings of the National Academy of Sciences of the United States of America* 105, 5459-5464.
- Goehring, A.S., Rivers, D.M., and Sprague, G.F., Jr. (2003). Urmlylation: a ubiquitin-like pathway that functions during invasive growth and budding in yeast. *Molecular biology of the cell* 14, 4329-4341.
- Kunzler, M., and Hurt, E.C. (1998). Cse1p functions as the nuclear export receptor for importin alpha in yeast. *FEBS letters* 433, 185-190.
- Kutay, U., Bischoff, F.R., Kostka, S., Kraft, R., and Gorlich, D. (1997). Export of importin alpha from the nucleus is mediated by a specific nuclear transport factor. *Cell* 90, 1061-1071.
- Lian, F.M., Yu, J., Ma, X.X., Yu, X.J., Chen, Y., and Zhou, C.Z. (2012). Structural Snapshots of Yeast Alkyl Hydroperoxide Reductase Ahp1 Peroxiredoxin Reveal a Novel Two-cysteine Mechanism of Electron Transfer to Eliminate Reactive Oxygen Species. *The Journal of biological chemistry* 287, 17077-17087.
- Miyamoto, Y., Saiwaki, T., Yamashita, J., Yasuda, Y., Kotera, I., Shibata, S., Shigeta, M., Hiraoka, Y., Haraguchi, T., and Yoneda, Y. (2004). Cellular stresses induce the nuclear accumulation of importin alpha and cause a conventional nuclear import block. *The Journal of cell biology* 165, 617-623.
- Nguyen, A.N., and Shiozaki, K. (1999). Heat-shock-induced activation of stress MAP kinase is regulated by threonine- and tyrosine-specific phosphatases. *Genes & development* 13, 1653-1663.
- Petersen, J., and Nurse, P. (2007). TOR signalling regulates mitotic commitment through the stress MAP kinase pathway and the Polo and Cdc2 kinases. *Nature cell biology* 9, 1263-1272.
- Quinn, J., Findlay, V.J., Dawson, K., Millar, J.B., Jones, N., Morgan, B.A., and Toone, W.M. (2002). Distinct regulatory proteins control the graded transcriptional response to increasing H<sub>2</sub>O<sub>2</sub> levels in fission yeast *Schizosaccharomyces pombe*. *Molecular biology of the cell* 13, 805-816.
- Rodriguez-Gabriel, M.A., Watt, S., Bahler, J., and Russell, P. (2006). Upf1, an RNA helicase required for nonsense-mediated mRNA decay, modulates the transcriptional response to oxidative stress in fission yeast. *Molecular and cellular biology* 26, 6347-6356.
- Shiozaki, K., and Russell, P. (1996). Conjugation, meiosis, and the osmotic stress response are regulated by Spc1 kinase through Atf1 transcription factor in fission yeast. *Genes & development* 10, 2276-2288.
- Shiozaki, K., Shiozaki, M., and Russell, P. (1998). Heat stress activates fission yeast Spc1/Styl MAPK by a MEKK-independent mechanism. *Molecular biology of the cell* 9, 1339-1349.
- Solsbacher, J., Maurer, P., Bischoff, F.R., and Schlenstedt, G. (1998). Cse1p is involved in export of yeast importin alpha from the nucleus. *Molecular and cellular biology* 18, 6805-6815.



Takada, H., Nishimura, M., Asayama, Y., Mannse, Y., Ishiwata, S., Kita, A., Doi, A., Nishida, A., Kai, N., Moriuchi, S., *et al.* (2007). Atf1 is a target of the mitogen-activated protein kinase Pmk1 and regulates cell integrity in fission yeast. *Molecular biology of the cell* 18, 4794-4802.

Umeda, M., Izaddoost, S., Cushman, I., Moore, M.S., and Sazer, S. (2005). The fission yeast *Schizosaccharomyces pombe* has two importin- $\alpha$  proteins, Imp1p and Cut15p, which have common and unique functions in nucleocytoplasmic transport and cell cycle progression. *Genetics* 171, 7-21.

Yarian, C., Townsend, H., Czestkowski, W., Sochacka, E., Malkiewicz, A.J., Guenther, R., Miskiewicz, A., and Agris, P.F. (2002). Accurate translation of the genetic code depends on tRNA modified nucleosides. *The Journal of biological chemistry* 277, 16391-16395.

Zhou, X., Ma, Y., Sugiura, R., Kobayashi, D., Suzuki, M., Deng, L., and Kuno, T. (2010). MAP kinase kinase kinase (MAPKKK)-dependent and -independent activation of Sty1 stress MAPK in fission yeast. *The Journal of biological chemistry* 285, 32818-32823.

## Chapter 5. Roles and regulation of Ubl conjugation pathways in autophagy.

### 5.1. Introduction

Autophagy is a cellular process that allows eukaryotic cells to break down their own proteins and organelles. During autophagy, cellular components are sequestered inside a double-membrane vesicle, the autophagosome, and transported to vacuoles or the lysosome. The autophagosome fuses with the vacuole/lysosome, releasing the inner vesicle into the lumen. This vesicle – now known as an autophagic body – is broken down enzymatically, releasing its constituents into the vacuole/lysosome, where they can be broken down by proteolytic enzymes (**Fig. 1.8**). Collectively, ubiquitin-directed proteasomal degradation and autophagy function in cellular ‘housekeeping’, as they allow the basal turnover of cellular proteins and organelles. However, although autophagy occurs under homeostatic conditions, it is important that it is upregulated in response to certain stimuli. For example, autophagy can provide alternative nitrogen and carbon sources during starvation by breaking down cellular components (see Section 1.1.6.3).

The molecular basis for autophagy has been extensively-characterised in *S. cerevisiae*. As part of the autophagosome formation mechanism, two different UbIs are conjugated to specific substrates. The first Ubl, Atg12, is conjugated to the Atg5 protein (Mizushima et al., 1998), whilst the second Ubl, Atg8, is conjugated to PE lipids of the growing autophagosome (Ichimura et al., 2000; Kirisako et al., 2000). The Atg12 and Atg8 conjugation pathways, and the links between them, have been described previously (**Fig. 1.9** and Section 1.1.6.2). Interestingly, the Atg8-specific ULP, Atg4, is oxidised and inactivated during oxidative stress in mammalian cells, leading to an increase in autophagic activity (Scherz-Shouval et al., 2007). The redox regulation of mammalian Atg4 agrees with numerous reports linking oxidative stress with the

induction of autophagy in higher eukaryotes (Scherz-Shouval and Elazar, 2007; Azad et al., 2009; Bensaad et al., 2009; Chen et al., 2007, 2008b; Huang et al., 2009). This finding also illustrates that overall autophagic activity can be regulated via autophagic Ubl conjugation. Thus, the Atg12 and Atg8 conjugation pathways represent an attractive regulatory target.

At the onset of this project, there had been very little characterisation of the molecular mechanisms underlying autophagy in *S. pombe*. Furthermore, it was largely unclear how the process was regulated. Moreover, the possibility that the autophagic E1, Atg7, and the E2s, Atg10 and Atg3, are redox-regulated has not been investigated, and redox regulation of Atg4 has not been reported in lower organisms. Given that other ubiquitin/Ubl conjugation pathways are redox-regulated in lower organisms, and that there are numerous links between stress and the induction of autophagy, it is possible that redox regulation of enzymes functioning in autophagic Ubl conjugation allows cells to modulate autophagic activity during oxidative stress. Hence, in this chapter we investigate fission yeast autophagic Ubl conjugation pathways, their roles in responses to oxidative stress, and how they may be redox-regulated in *S. pombe*. These studies have also identified a putative Atg10 homologue which does not appear to function in autophagy; instead, this enzyme is implicated in responses to a range of stresses in *S. pombe*.

## **5.2. Investigations of redox regulation of enzymes functioning in autophagic Ubl conjugation, and possible links between ROS and autophagy in *S. pombe*.**

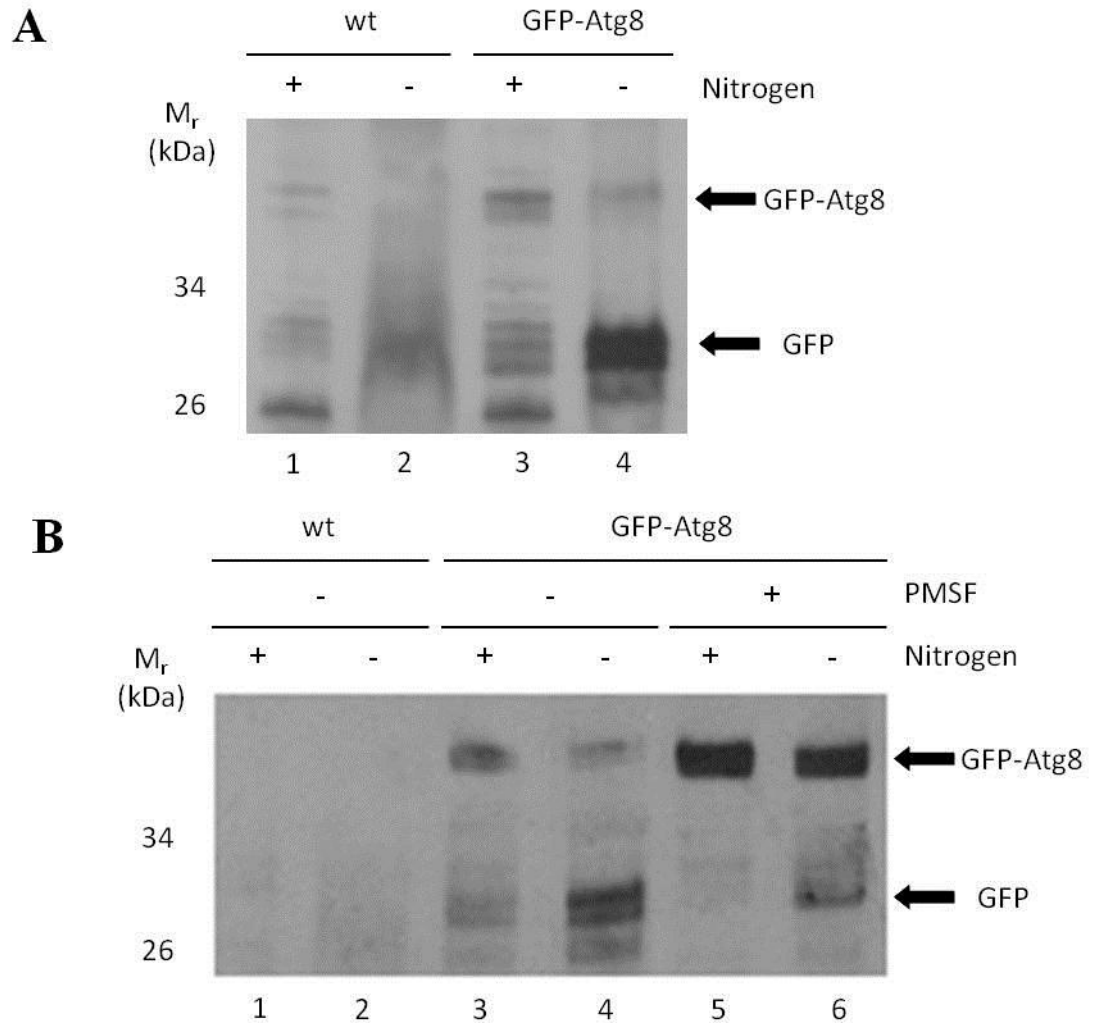
### **5.2.1. Development of a GFP-release assay to allow studies of autophagy in *S. pombe*.**

At the onset of these investigations, very few studies of fission yeast autophagy had been reported. Consequently, there was no standard assay available to monitor

autophagic activity in *S. pombe*. Therefore, it was important to design an assay for autophagic activity in fission yeast. Subsequently, the effect of various stress conditions and gene mutations on autophagic activity could be investigated.

Atg8 is conjugated to PE lipids on the surface of the growing autophagosome (Ichimura et al., 2000; Kirisako et al., 2000). Following fusion of the autophagosome and the yeast vacuole, Atg8 is released into the lumen of the vacuole and broken down by proteolytic enzymes. Previous work has shown that when a GFP-Atg8 fusion protein is expressed in *S. cerevisiae*, Atg8 breakdown releases free GFP into the cell, which can be monitored by western blotting (Klionsky et al., 2007). However, mutation of *atg* gene(s) prevents this breakdown, indicating that GFP release is representative of autophagic activity (Klionsky et al., 2007). Another group have previously generated a strain expressing a GFP-Atg8 fusion protein from the normal *atg8*<sup>+</sup> locus in *S. pombe* (Kohda et al., 2007). Hence, a GFP release assay for autophagy in *S. pombe* was developed using this GFP-Atg8 strain (JT268; gift from M. Yamamoto). Significantly, this strain lacks auxotrophic markers, allowing the effects of nitrogen starvation on autophagy to be investigated. GFP-Atg8 cells were incubated in standard minimal medium (EMM) or nitrogen-free medium (EMM-N) to induce autophagy, and protein extracts were prepared. GFP-Atg8 breakdown was then examined by western blotting, using anti-GFP antibodies. In contrast to *S. cerevisiae* and mammalian cells, autophagy does not appear to be rapidly induced in *S. pombe* when cells are transferred to nitrogen-free medium. Indeed, free GFP was not detected in protein extracts if cells had been growing in nitrogen-free medium for less than 4 hours (data not shown). However, if cells were incubated in nitrogen-free medium for over 18 hours, free GFP was readily detectable using western blotting (**Fig. 5.1A**). When cells were grown in nitrogen-replete medium, GFP-Atg8 was detectable, but much less free GFP was present in protein extracts (**Fig. 5.1A, compare lanes 3 and 4**). Noticeably, the level of free GFP detected in extracts from cells grown in nitrogen-free medium was far greater than the levels of GFP-Atg8 detected in extracts from cells grown in nitrogen-replete medium (**Fig. 5.1A, compare lanes 3 and 4**). These data suggest that free GFP accumulates in the cell following proteolysis of GFP-Atg8, and that *atg8*<sup>+</sup> expression could be increased during nitrogen starvation.

Despite these encouraging results, it remained possible that GFP-Atg8 could be hydrolysed independently of autophagy during nitrogen starvation. Fusion of the



**Figure 5.1. Design and validation of a GFP release assay for autophagy in *S. pombe*.**

**A.** Cells lacking auxotrophic markers and expressing Atg8 tagged with GFP at the N-terminus (GFP-Atg8; JT268) and wild-type (wt; SW576) cells were incubated in minimal medium (EMM) or minimal medium lacking a nitrogen source (EMM-N) for 20 hours at 30°C. Extracts were prepared and analysed by western blotting, using anti-GFP antibodies. GFP-Atg8 (~45kDa) and free GFP released during autophagy (~30kDa) are indicated. This experiment was repeated multiple times, giving the same result each time. **B.** GFP-Atg8 and wild-type (wt) cells were incubated in EMM or EMM-N as above, with or without 1mM PMSF in the media. Cellular extracts were prepared and analysed as above. This experiment was repeated twice, giving the same result each time.

autophagosome and the vacuole allows vacuolar proteases to break down proteins previously contained in the autophagosome, including Atg8. Hence, if GFP release is entirely attributable to autophagy, this could be prevented by inhibiting vacuolar proteases. Thus, to investigate whether GFP release is a result of GFP-Atg8 delivery to the vacuole, cells were grown in nitrogen-free medium in the presence or absence of the vacuolar protease inhibitor PMSF, which has previously been shown to inhibit autophagy in *S. pombe* (Kohda et al., 2007). As expected, GFP-Atg8 levels decreased and levels of free GFP were greatly increased following nitrogen starvation (**Fig. 5.1B, compare lanes 3 and 4**). In contrast, GFP-Atg8 hydrolysis was almost completely blocked in the presence of PMSF (**Fig. 5.1B, compare lanes 4 and 6**). Hence, these data indicate that the majority of GFP-Atg8 hydrolysis is a result of increased delivery to the vacuole via autophagosomes. Furthermore, PMSF prevents GFP-Atg8 breakdown even when cells are grown in medium containing a nitrogen source (**Fig. 5.1B, compare lanes 3 and 5**). This suggests that autophagy is occurring at a low basal level in *S. pombe*, as in other eukaryotes. Furthermore, monitoring GFP-Atg8 hydrolysis appears to be a good measure of autophagic activity in *S. pombe*, as in other organisms, and these results support previous findings that longer periods of nitrogen starvation are required to induce autophagy in *S. pombe* than in other organisms (Kohda et al., 2007).

Next, to confirm that the Atg12/Atg8 conjugation pathway is required for autophagy in *S. pombe*, GFP release during nitrogen starvation was investigated in  $\Delta atg12$  cells. The *atg12*<sup>+</sup> gene is essential for autophagy in *S. cerevisiae* (Tsukada and Ohsumi, 1993). Furthermore, Atg12 is the Ubl required for the conjugation of Atg8 to PE in *S. cerevisiae* (Ichimura et al., 2000; Kirisako et al., 1999; Kirisako et al., 2000), and is therefore central to investigations of autophagic Ubl conjugation pathways. To identify fission yeast Atg12, BLAST searches of the *S. pombe* proteome were performed, using the amino acid sequence of *S. cerevisiae* Atg12 (**Fig. 5.2A**). The fission yeast homologue of Atg12 is a protein with a M<sub>r</sub> of ~15kDa, and is encoded by a gene on chromosome I (*SPAC1783.06c*) now designated *atg12*<sup>+</sup>. A  $\Delta atg12$  strain was constructed using the PCR-based technique described previously (**Fig. 3.10A**), using gDNA from a strain (BG\_0465) in which *atg12*<sup>+</sup> is replaced by a *KanMX6* cassette. Incorporation of *KanMX6* at the *urml*<sup>+</sup> locus in a strain lacking auxotrophic markers (SW576) was confirmed by PCR (**Fig. 5.2B**). To construct GFP-Atg8  $\Delta atg12$ , this  $\Delta atg12$  strain was crossed with the GFP-Atg8 strain used previously (JT268). Next, to examine the role of Atg12 in autophagy, GFP-Atg8 and GFP-Atg8  $\Delta atg12$  cells were

**A**

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ScAtg12      MSRIESENETESDESSIISTNNGTAMERSRNNQELRSSPHTVQNRLELFSRRLSQLGLA 60
SpAtg12      -----MTGLVDQQELEKRLATEED----- 19
                  *      :;***      * :;

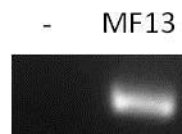
ScAtg12      SDISVDQQVEDSSSGTYEQEETIKTNAQTSKQKSHKDEKNIQKIQKFQPIGSIGQLKPS 120
SpAtg12      ---SQNEDIEN-----QLPSIETIINTYREKENR-----RVNLRFKAIGRTPLLRKT 63
                  * :;::*:      * :;::* :; * :;::* :; :;::*: ** * :;

ScAtg12      VCKISMSQSFAMVILFLKRRLLKMDH---VYCYINNSFAPSQQNIGELWMQFKTNDELIV 177
SpAtg12      VFSINASQRFKQVTRFLKKELGLPMNSSLVLYVNSSFAPS PDEIVGNLYDNFAIDSHLLI 123
                  * * ** * * ***: * :      : *;* *****: :;*: * : * :;

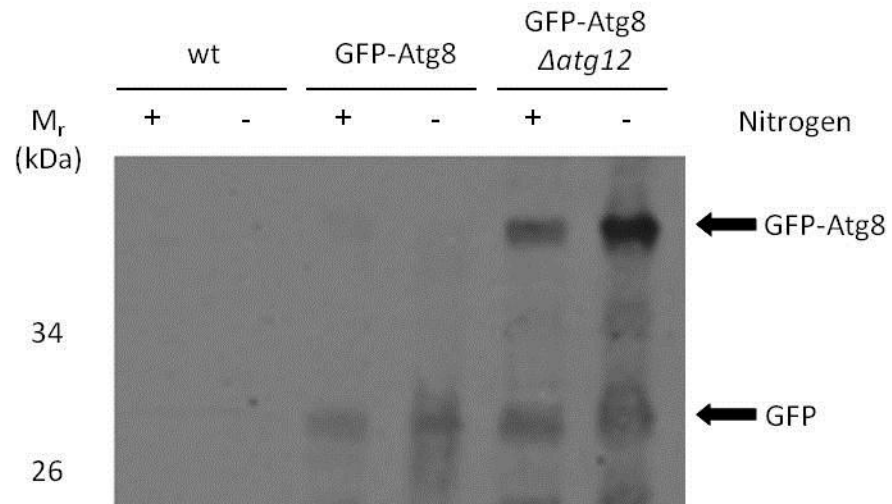
ScAtg12      SYCASVAFG 186
SpAtg12      NYCINVAFG 132
                  ** *****

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**B**



**C**



**Figure 5.2. Mutation of the autophagic Ubl conjugation pathway inhibits autophagy in *S. pombe*.**

**A.** The fission yeast homologue of *S. cerevisiae* (Sc) Atg12 was identified by BLAST search of the *S. pombe* (Sp) proteome, with 32% identity. An amino acid sequence alignment is shown above. Identical residues are indicated by asterisks, and similar residues are indicated by colons. **B.** A  $\Delta atg12$  strain was constructed using the technique described in Fig. 3.10A, using the oligonucleotide primers Atg12delf and Atg12delr (see Table 2.2) and gDNA from BG\_0465 as a template for PCR. To confirm  $\Delta atg12$  strain construction, gDNA was extracted from a G-418-resistant colony (MF13), as well as wild-type (SW576; -) cells. PCR was performed using these gDNA extracts as templates, using the forward primer Atg12delf and the reverse primer KanMX6delchk, which binds to a site within *KanMX6* (see Table 2.2). PCR products were analysed by agarose gel electrophoresis, and a PCR product of ~500bp confirmed strain construction. **C.** GFP-Atg8 (JT268), GFP-Atg8  $\Delta atg12$  (MF43), and wild-type (wt; SW576) cells lacking auxotrophic markers were incubated in EMM or EMM-N for 20 hours at 30°C. Cellular extracts were prepared, and GFP release from the GFP-Atg8 fusion protein was investigated by western blotting using anti-GFP antibodies. GFP-Atg8 (~45kDa) and free GFP released during autophagy (~30kDa) are indicated. This experiment was repeated twice with the same result.

incubated in nitrogen-free medium for 20 hours. Cellular extracts were prepared, and GFP release was analysed using western blotting. When *atg12*<sup>+</sup> was mutated, GFP-Atg8 breakdown was significantly impaired (**Fig. 5.2C**). This again indicates that autophagy is required for the breakdown of GFP-Atg8, and is consistent with Atg12 having an essential role in autophagy in *S. pombe*. Noticeably, some free GFP was also detected in GFP-Atg8  $\Delta$ *atg12* cellular extracts, again suggesting that GFP-Atg8 can be broken-down independently of autophagy. However, autophagy-independent GFP-Atg8 breakdown appears to be relatively minimal. Therefore, monitoring GFP-Atg8 breakdown remains an effective tool for investigating autophagy in *S. pombe*.

### **5.2.2. Identification and epitope-tagging of potential fission yeast homologues of Atg7, Atg4, Atg3, and the identification of a putative Atg10 homologue.**

Having successfully developed an assay for autophagy in *S. pombe*, the next step was to investigate possible redox regulation of Atg12/Atg8 conjugation, and how this may impact on autophagy as a whole. First, to identify fission yeast homologues of Atg7, Atg4, Atg10 and Atg3, BLAST searches of the *S. pombe* proteome were performed, using the amino acid sequences of the equivalent proteins in *S. cerevisiae*. This analysis successfully identified homologues of Atg7, Atg4 and Atg3. Fission yeast Atg7 is a ~70kDa protein encoded by a gene on chromosome II (*SPBC6B1.05c*), and is 47% identical to *S. cerevisiae* Atg7 (**Fig. 5.3A**). Atg4 has a M<sub>r</sub> of ~35kDa, is encoded by a gene on chromosome I (*SPAC19B12.08*), and is 37% identical to the *S. cerevisiae* protein (**Fig. 5.3B**). Fission yeast Atg3 is a ~30kDa protein, is 33% identical to the homologue in *S. cerevisiae*, and is encoded by a gene on chromosome II (*SPBC3B9.06c*) (**Fig. 5.3C**).

Curiously, the *S. pombe* homologue of Atg10 could not be identified by BLAST search of the fission yeast proteome. However, *S. cerevisiae* Atg3 and Atg10 have similar amino acid sequences at their catalytic sites which are unique to autophagic E2s (Ichimura et al., 2000; Geng and Klionsky, 2008). The catalytic cysteines of Atg3 and Atg10 form part of a ΨHPC motif (where Ψ represents a hydrophobic residue). Hence, to attempt to identify the Atg10 enzyme in *S. pombe*, the fission yeast proteome was



[illegible]

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## B

ScAtg4	M-----QRWLQLWKMDLVQKVSHGVFEGSSSEPAALMNHDIYVLGEVYPERDEESGAE
SpAtg4	MELMARFLERYLHFAPT-----TEPPGTLI-----
	* :*: *: : : * * :*:
ScAtg4	QCEQDCRYRGEAVSDGFLSSLFGREISSYTKEFLLDVQSRVNFYRTRFVPIARAPDGPS
SpAtg4	-----WFLGHSYKIEDSQWPEKFLYDSFSLITITYRSG-----
	** : * * : : ** * * : : ***:
ScAtg4	PLSLNLLVRTNPISTIEDYIANPDCFNTDIGWGCMTGTGQSLLGNALQILHLGRDFRVNG
SpAtg4	-----IEGLENMTSDTGWGCMIRSTQTLLANCLRICYPEKQLK---
	* : : : * *****: * : ** * * : : : :
ScAtg4	NESLERESKFVNWFNDTPEAPFLHNFSAGTELSDKRPGEWFGPAATARSIQSLIYGFP
SpAtg4	-----EILALFADEPSAPFSIHQFVTMGKTLCDINPGQWFGPTTSCSCVARLSDQNP
	: : : * * * *****: * : * * * ** : *****: : : : * *
ScAtg4	ECGIDDCIVSVSSGDIYENEVEKVFAENPNRILFLLGVKLGINAVNESYRESICGILSS
SpAtg4	DVPL-HVYVARNGNAIYRDQLSKV-----SFPVLLLIPTRLGIDSINESYYDQLQVFEI
	: : : * : ** : : : ** : * : : : ***: : ***** : : : :
ScAtg4	TQSVGIAGGRPSSSLYFFGYQGNEFLHFDPH-----IPQPAVEDSFVESCHTSKFGKL
SpAtg4	RSFVGITGGRPRSAHYFYARQNQYFFYLDPHCTHFAHTTTQPAEETF-----HSATLRRV
	***: ***** * : * : : * : : : : *** ***** * : * * : : : :
ScAtg4	QLSEMDPSMLIGILIKGEKDWWQWKLEVAESA-IINVL-----AKRMDDFDVSCSMDDV
SpAtg4	AIQDLDPCEMIFGFLIRDEEWHSEANQKYFADIVQIFDSEPQPVETHDDFVLDENVED-
	: : : ** * : : * : * : : : : * * : : : : : ***** : : * :
ScAtg4	ESVSSNSMKKASNNENLGVLEGDYVDIGAIFPHTTNTEDVDEYDCFQDIHCKKQKIVVM
SpAtg4	-----
ScAtg4	GNTHIVNANLTDYEVEGLVEKETVGIHSPIDEKCx
SpAtg4	-----H-----L
	*

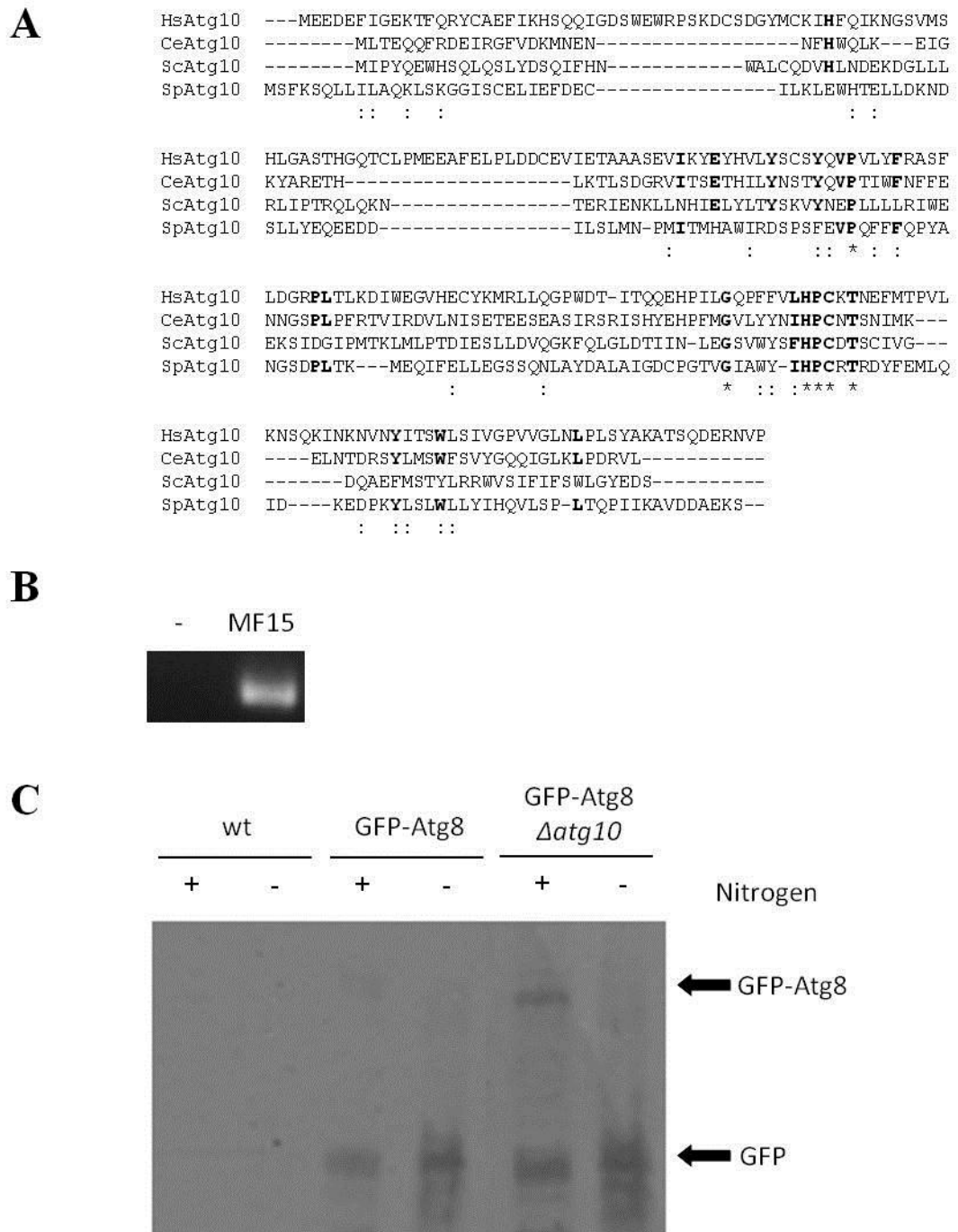
## C

ScAtg3	M---IRSTLSSWREYLTPITHKSTFLTGTGQITPEEFVQAGDYLCHMFPTWKWNEESSDIS
SpAtg3	MAQRLTSAFLNWRHITPASKTSDFENTGMISPEEFVLAGDYLVSKEPTWSW--ECGD-R
	* : * : : ***: * : : * * * * : ***** ***** ***** * * *
ScAtg3	YRDFLPKNKQFLIIRKVPCDKR-----AEQCVEVEGPDVIMKGFAEDGEDDVLEYIGS
SpAtg3	IRGFLPKDKQYLVRHVFCVQRNINIGVNEEWVDIETDDTRNK---DDDQDDDAISSIHS
	* *****: * : * : * * * * : * : * : * * * : * : * : * : *
ScAtg3	ETEHVQSTPAGGTDSSIDDIDELIQDMEIKEEDENDDTEEFNAKGGLAKDMAQERYIDL
SpAtg3	DTSDIASAERLKGQSKELSDSGPL----PLKDEEDDDQMVSPViked-----EGRYIDL
	: * : * : : : : * * : * : * : : * : *****
ScAtg3	YIAYSTS YRVPKMYIVGFNSNGSPLSPEQMFEDISADYRTKTATIEKLPFYKNSVLSVSI
SpAtg3	YIVYDKYRTPRFLRGWAGGQLLTMKDIYEDVSGEHAGKTVMTEPPPHYHSHNTMASV
	** * * * * : : : : * : * * : : : * : * : : * * * : * : *
ScAtg3	HPCKHANVMKILLDKVRVVRQRRRKELQEEQELDGVGDWEDLQDDIDDSLVRDQYLIVFL
SpAtg3	HPCKHASVL-----LKLKQHRER-----NDPIRVDQYMYLFL
	***** * : : : : * : * : : * : : * : *****: * :
ScAtg3	KFITSVTPSIQHDYTMEGW
SpAtg3	KFVSTMLPYFEIDYTIQA-
	** : : : * : : *****:

scanned for proteins that included the ΨHPC motif. 26 proteins were found to contain this motif (**Table 5.1**), and Atg3 is one of these proteins. Of the remaining 25 proteins, 15 are encoded by genes that have been characterised previously and do not function in ubiquitin/Ubl conjugation, and another seven have significant homology to proteins which have not been linked to ubiquitin/Ubl conjugation. Of the final three proteins, one has a predicted  $M_r$  of ~20kDa, which is consistent with its possible function as an E2, and very similar to the  $M_r$  of budding yeast Atg10. Furthermore, this protein includes many amino acids that are also present in Atg10 homologues in other organisms (**Fig. 5.4A**). This protein is encoded by a gene on chromosome I (*SPAC227.04*) and will be referred to as SpAtg10. To investigate whether SpAtg10 is involved in autophagy, the strategy outlined in **Fig. 3.10A** was used to construct a *Δatg10* strain lacking auxotrophic markers, using a Bioneer strain (BG\_0880) with a *KanMX6* cassette at the *atg10*<sup>+</sup> locus. Incorporation of *KanMX6* at the *atg10*<sup>+</sup> locus of SW576 cells was confirmed by PCR (**Fig. 5.4B**). Next, a GFP-Atg8 *Δatg10* strain was constructed by genetic cross between the *Δatg10* strain (MF15) and the GFP-Atg8 strain used previously (JT268). To investigate the potential role of SpAtg10 in autophagy, GFP-Atg8 *Δatg10* cells were incubated in nitrogen-free medium for 20 hours. Cellular extracts were prepared, and GFP-Atg8 breakdown was analysed by western blotting. Unexpectedly, GFP release was not prevented in cells lacking SpAtg10 (**Fig. 5.4C**), suggesting that SpAtg10 is not required for autophagy. This is particularly significant given that Atg12 is required for autophagy (**Fig. 5.2C**), and Atg12 is the predicted cognate Ubl of SpAtg10. Thus, autophagic Ubl conjugation pathways in *S. pombe* may not follow the same pattern as those of *S. cerevisiae* (**Fig. 1.9**). The function of SpAtg10 will be investigated later (see Section 5.3).

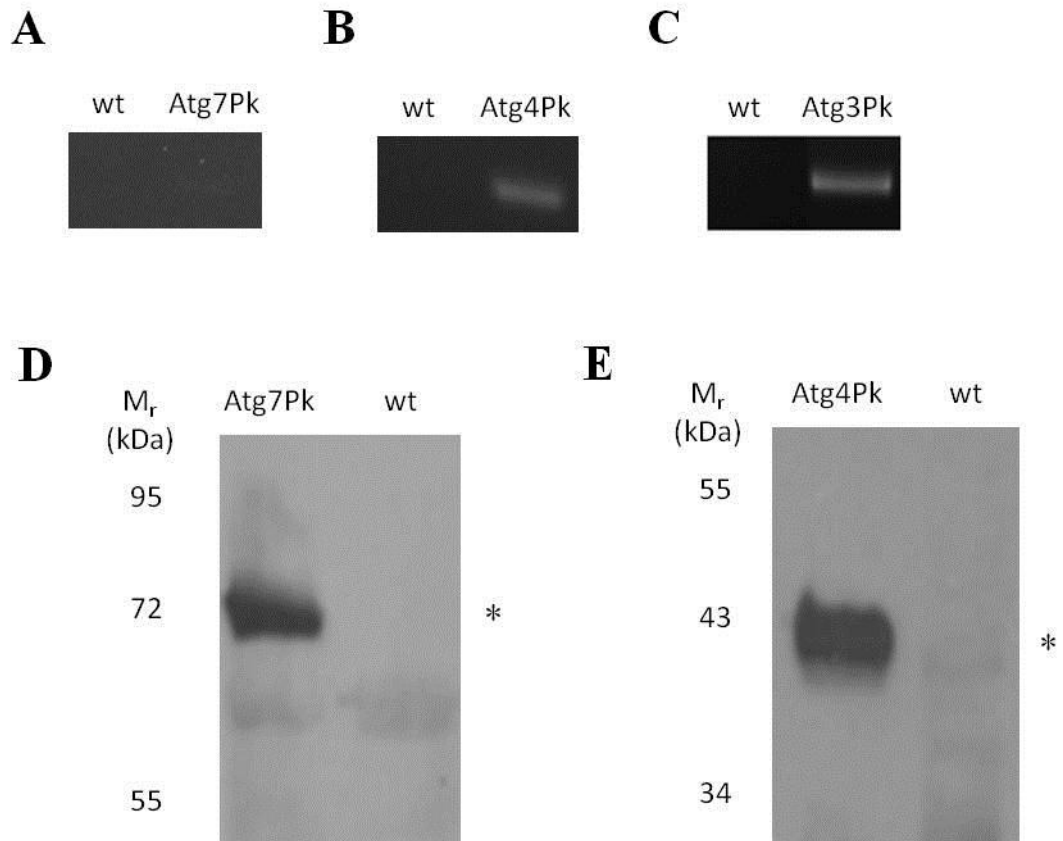
No antibodies specific for Atg7, Atg4 or Atg3 are commercially available. Hence, in order to investigate redox regulation of these proteins, it was necessary to construct strains expressing Pk epitope-tagged proteins from normal chromosomal loci, using the tagging strategy described in **Fig. 3.2** and the integration tagging plasmids pRIP42PkC-*atg7*, pRIP42PkC-*atg4* and pRIP42PkC-*atg3* (see Section 2.1.5.1). These plasmids were linearised and introduced into cells, allowing homologous recombination and incorporation into the genome at the appropriate loci. The incorporation of the correct plasmid at each locus was confirmed by PCR (**Fig. 5.5A-C**), and western blotting was used to confirm that Pk epitope-tagged proteins were stably-expressed. However, although Atg7Pk (**Fig. 5.5D**) and Atg4Pk (**Fig. 5.5E**) could be detected, Atg3Pk could

Table 5.1



**Figure 5.4. A putative *S. pombe* Atg10 is not required for autophagy.**

**A.** A sequence alignment showing the amino acid sequences of Atg10 homologues in humans (Hs), *C. elegans* (Ce), *S. cerevisiae* (Sc), and the putative Atg10 in *S. pombe* (Sp). Identical residues are indicated by asterisks, and similar residues are indicated by colons. Amino acid residues present in multiple homologue are emboldened. **B.** A  $\Delta atg10$  strain was constructed using the technique described in Fig. 3.10A, using the oligonucleotide primers Atg10delf and Atg10delr (see Table 2.2) and gDNA from BG\_0880 as a template for PCR. To confirm  $\Delta atg10$  strain construction, gDNA was extracted from a G-418-resistant colony (MF15), as well as wild-type (SW576; -) cells. PCR was performed using these gDNA extracts as templates, using the forward primer Atg10delf and the reverse primer KanMX6delchk, which binds to a site within *KanMX6* (see Table 2.2). PCR products were analysed by agarose gel electrophoresis, and a PCR product of ~500bp confirmed strain construction. **C.** GFP-Atg8 (JT268), GFP-Atg8  $\Delta atg10$  (MF31) and wild-type (SW576) cells lacking auxotrophic markers were incubated in EMM or EMM-N for 20 hours at 30°C. To investigate autophagic activity, cellular extracts were prepared, and GFP release from the GFP-Atg8 fusion protein was investigated by western blotting using anti-GFP antibodies. This experiment was repeated twice with the same result.



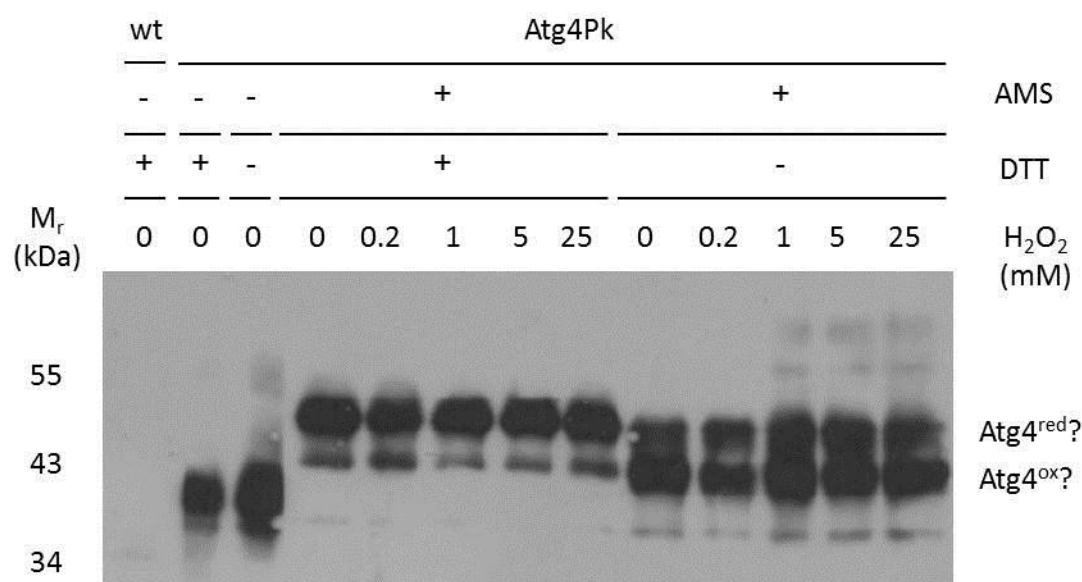
**Figure 5.5. Pk epitope-tagging of Atg 7, Atg 4 and Atg 3 at their C-termini.**

A-C. To confirm plasmid integration, gDNA was extracted from wild-type (wt; CHP428) cells and from cells transformed with linearised (A) pRIP42PkC-*atg7* to tag Atg7 with Pk epitopes (Atg7Pk; MF01), (B) pRIP42PkC-*atg4* to tag Atg4 with Pk epitopes (Atg4Pk; MF03) and (C) pRIP42PkC-*atg3* to tag Atg3 with Pk epitopes (Atg3Pk; MF09). Integration at the correct loci was confirmed by PCR using the forward primers (A) Atg7chkf, (B) Atg4chkf and (C) Atg3chkf, which bind to sites within the respective ORFs, and the reverse primer nmtend (see **Table 2.2**), and using gDNA extracts as templates. PCR products were analysed by agarose gel electrophoresis, and products of (A) ~750bp, (B) ~400bp and (C) ~500bp were taken as confirmation of plasmid integration. D, E. Extracts from (D) Atg7Pk (MF01) and (E) Atg4Pk (MF03) cells were prepared and analysed by western blotting. Extracts from wild-type (wt; CHP428) cells were also prepared, as controls. Pk epitope-tagging was confirmed using anti-Pk antibodies. Atg7Pk (~70kDa, as expected) and Atg4Pk (~40kDa, as expected) are indicated by asterisks.

not be detected in cellular extracts (data not shown). Interestingly, epitope-tagged Atg10 and Atg3 were only detected in *S. cerevisiae* following enrichment by immunoprecipitation (Shintani et al., 1999; Ichimura et al., 2000). This suggests that Atg3 may be expressed at such low levels in both *S. cerevisiae* and *S. pombe* that it is difficult to detect by western blotting. However, it is also possible that Pk epitope-tagging of Atg3 has decreased the stability of the protein, preventing its detection by western blotting.

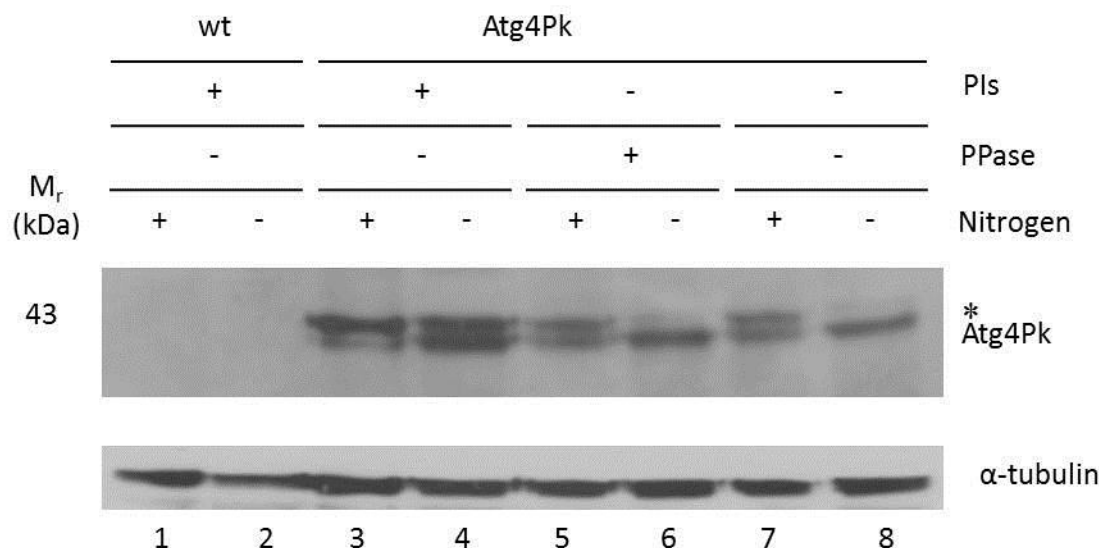
### **5.2.3. Atg4 does not appear to be redox-regulated in *S. pombe*, but the enzyme could be regulated by post-translational modification.**

While oxidative stress is associated with the induction of autophagy, there are very few examples of redox regulation of Atg proteins. However, mammalian Atg4 is inhibited by H<sub>2</sub>O<sub>2</sub> via oxidation of a key cysteine downstream of the catalytic residue, which may trigger intramolecular disulfide formation (Scherz-Shouval et al., 2007). This prevents the removal of Atg8 from the autophagosomal membrane, and leads to increased autophagic activity. Despite this, it is unclear whether redox regulation of Atg4 is a conserved mechanism in eukaryotes. Furthermore, the redox-sensitive cysteine residue of Atg4 is replaced by a serine or threonine residue in lower organisms such as *S. cerevisiae* and *S. pombe* (Scherz-Shouval et al., 2007), which may render Atg4 insensitive to oxidation in yeast. However, this possibility has not been investigated. Hence, to investigate cysteine oxidation in the *S. pombe* homologue, Atg4Pk cells were subjected to a range of concentrations of H<sub>2</sub>O<sub>2</sub>, and cellular extracts prepared by acid lysis to preserve the oxidation state of cysteine residues. Cellular extracts were treated with the alkylating agent AMS. As outlined previously, AMS transfers an alkyl group to reduced cysteines, but not oxidised cysteines. These extracts were examined by western blotting. Interestingly, Atg4 appears to be present in oxidised (Atg4<sup>ox</sup>) and reduced (Atg4<sup>red</sup>) forms (**Fig. 5.6, AMS+ DTT-**). A significant pool of Atg4 was found in the Atg4<sup>red</sup> form. However, the majority of cellular Atg4 was in the Atg4<sup>ox</sup> form. Significantly, the levels of Atg4<sup>ox</sup> are not increased by H<sub>2</sub>O<sub>2</sub>. These AMS-modification experiments suggest that fission yeast Atg4 is not redox-regulated by H<sub>2</sub>O<sub>2</sub>, in contrast to mammalian Atg4 (Scherz-Shouval et al., 2007). It is possible that Atg4 homologues lacking the redox-sensitive cysteine found in mammalian Atg4 are not redox-regulated.



**Figure 5.6. Atg4 is present in oxidised (Atg4<sup>ox</sup>) and reduced (Atg4<sup>red</sup>) forms, but the level of oxidised protein is not increased by H<sub>2</sub>O<sub>2</sub>.**

Atg4Pk (MF02) cells were treated with the indicated concentrations of H<sub>2</sub>O<sub>2</sub> for 20 minutes, and cellular extracts were prepared by TCA lysis. An extract from wild-type (wt; CHP429) cells expressing untagged protein was also prepared, as a control. During extraction, protein samples were treated with the cysteine reducing agent DTT and/or the cysteine alkylating agent AMS, as indicated. The oxidation state of Atg4 was analysed by western blotting, using anti-Pk antibodies. Atg4<sup>ox</sup> and Atg4<sup>red</sup> are indicated. This experiment was repeated twice with the same result.



**Figure 5.7. Atg4 phosphorylation is decreased following nitrogen starvation.**

Atg4Pk cells lacking auxotrophic markers (MF20) and wild-type (wt; SW576) cells were incubated in minimal medium (EMM) or minimal medium lacking a nitrogen source (EMM-N) for 20 hours at 30°C. Cellular extracts were prepared with or without phosphatase inhibitors (PIs) in the lysis buffer, as indicated. Extracts prepared without PIs were treated with λ-phosphatase (PPase), as indicated. Atg4Pk modification was then analysed by western blotting, using anti-Pk antibodies. β-tubulin levels are shown as a loading control. A slower mobility form of Atg4 is indicated by an asterisk. This experiment was repeated multiple times, giving the same result each time.



Interestingly, Atg4 was not detected as a single band following DTT treatment (**Fig. 5.6, AMS+ DTT+**), raising the possibility that the protein may be targeted by other modifications. Hence, the possibility that Atg4 modification is modulated during induction of autophagy was investigated. First, an Atg4Pk strain lacking auxotrophic markers (MF20) was generated by genetic cross with the SW576 strain. These Atg4Pk cells were grown in standard media and nitrogen-free media for 20 hours. To investigate the possibility that Atg4 is modified by phosphorylation, extracts were prepared in the presence or absence of phosphatase inhibitors, and extracts prepared in the absence of these inhibitors were treated with  $\lambda$ -phosphatase. These extracts were analysed by western blotting. When cells were grown in nitrogen-replete media, the majority of Atg4 was found to be present in a slower mobility form (**Fig. 5.7, lane 3**). Moreover, this slower mobility form may be phosphorylated Atg4, as omission of phosphatase inhibitors from the lysis buffer caused a decrease in the levels of the slower mobility form (**Fig. 5.7, compare lanes 3 and 7**), although treatment of extracts with  $\lambda$ -phosphatase did not reverse this effect (**Fig. 5.7, compare lanes 5 and 7**). Interestingly, nitrogen starvation decreased the levels of the putative phosphorylated Atg4 (**Fig. 5.7, compare lanes 3 and 4**), and levels of this form of Atg4 were decreased yet further in the absence of phosphatase inhibitors (**Fig. 5.7, compare lanes 4 and 8**). Taken together, these results suggest that Atg4 may be modified by phosphorylation, and that this modification is decreased during nitrogen starvation. It is possible that induction of autophagy involves either the dephosphorylation of Atg4, or the inhibition of a kinase that phosphorylates Atg4 under homeostatic conditions. How this affects the function of Atg4 remains unclear, and requires further investigation.

#### **5.2.4. Fission yeast Atg7 does not form HMW disulfide complexes during oxidative stress.**

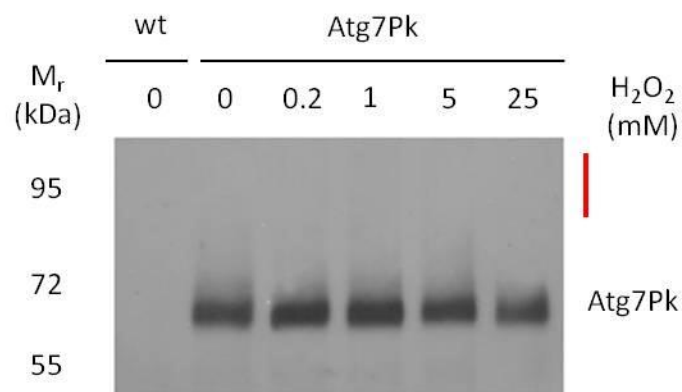
Given that other E1s form HMW disulfide complexes following oxidation of their catalytic cysteine residues, and having constructed a strain expressing Pk epitope-tagged Atg7, the possibility that Atg7 forms inhibitory HMW disulfide complexes during oxidative stress was investigated. Atg7Pk cells were treated with a range of H<sub>2</sub>O<sub>2</sub> concentrations, and cellular extracts prepared and analysed by western blotting. Interestingly, Atg7Pk did not form any HMW complexes, regardless of the

concentration of H<sub>2</sub>O<sub>2</sub> (**Fig. 5.8**), suggesting that autophagic Ubl conjugation is not inhibited by H<sub>2</sub>O<sub>2</sub> via this mechanism in *S. pombe*. This was not unexpected, given that oxidative stress has often been associated with the induction of autophagy, rather than inhibition (see Section 1.1.6.4).

Pk epitope-tagged Atg3 expressed from the normal chromosomal locus could not be detected in cellular extracts (data not shown). Hence, it was possible that Atg7 does form HMW complexes with Atg3 following oxidative stress, but that these complexes are undetectable by western blot analysis due to low levels of Atg3. To investigate the possibility that HMW complexes could be detectable if Atg3 levels were increased, the pREP41-*atg3* expression plasmid (see Section 2.1.5.2) was constructed to allow overexpression of *atg3*<sup>+</sup> using the *nmt* promoter (**Fig. 5.9**). Next, pREP41-*atg3* was introduced into Atg7Pk cells, which were then treated with a range of H<sub>2</sub>O<sub>2</sub> concentrations. Cellular extracts were then prepared and analysed by western blotting. Similar to the analysis of cells expressing Atg3 from the normal chromosomal locus, Atg7Pk did not form HMW complexes when cells containing pREP41-*atg3* were treated with H<sub>2</sub>O<sub>2</sub> (**Fig. 5.10**). This result suggests that Atg7 and Atg3 do not form HMW disulfide complexes during oxidative stress in *S. pombe*, although the predicted increases in Atg3 levels due to expression from pREP41-*atg3* were not tested. Interestingly, expression of *atg3*<sup>+</sup> from pREP41-*atg3* results in the appearance of a slower mobility form of Atg7 (**Fig. 5.10**). The nature of this slower mobility form of Atg7 is unclear. However, it is too small a change in mobility to be an Atg7-E2 complex or an Atg7-Ubl thioester. It is possible that Atg7 is post-translationally modified when Atg3 levels are increased, thus decreasing the mobility of Atg7Pk.

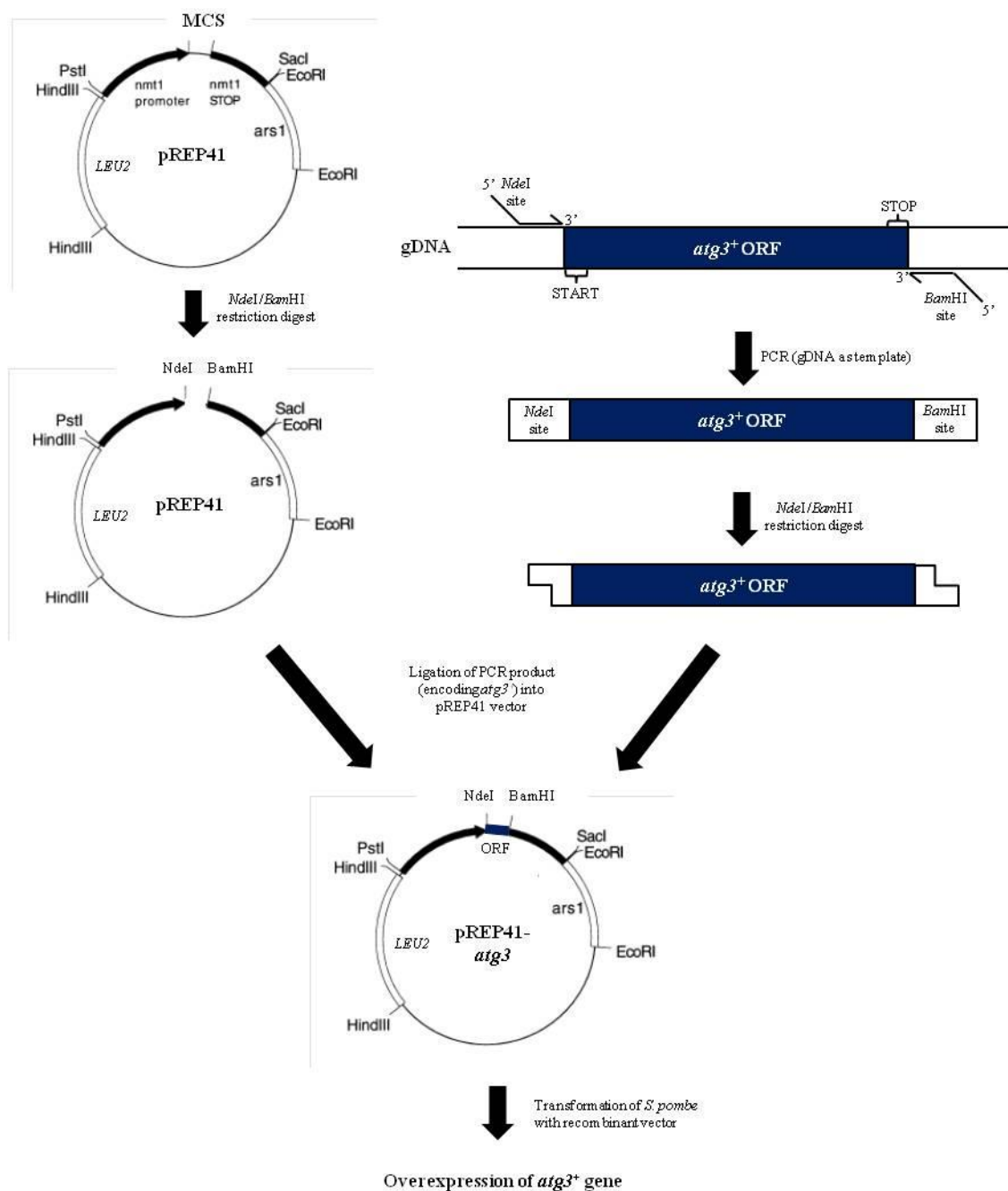
#### **5.2.5. Investigation of links between autophagy and responses to H<sub>2</sub>O<sub>2</sub> in *S. pombe*.**

There are many reports of increased autophagic activity during oxidative stress in eukaryotic cells (Scherz-Shouval and Elazar, 2007; Azad et al., 2009; Bensaad et al., 2009; Chen et al., 2007, 2008b; Huang et al., 2009), implicating autophagy in responses to oxidative stress. Interestingly, fission yeast *atg* genes are required for resistance to paraquat (Mikawa et al., 2010), a chemical which increases mitochondrial ROS



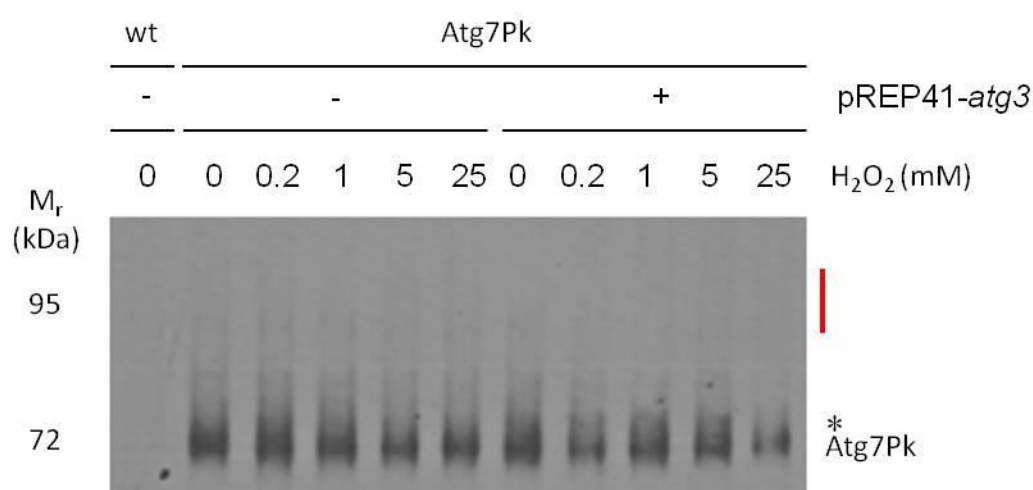
**Figure 5.8. Atg7 does not form HMW complexes during  $H_2O_2$ -induced oxidative stress in *S. pombe*.**

Atg7Pk (MF01) cells were treated with the indicated concentrations of  $H_2O_2$  for 20 minutes, and protein extracts prepared by TCA lysis. An extract from wild-type (wt; CHP428) cells expressing untagged protein was used as a control. Protein extracts were analysed by western blotting under non-reducing conditions, using anti-Pk antibodies. The position on the blot including proteins of the  $M_r$  expected for E1-E2 disulfide complexes is indicated by a red line. This experiment was repeated twice with the same result.



**Figure 5.9. The *atg3<sup>+</sup>* gene was overexpressed using the pREP41 expression vector.**

The *atg3<sup>+</sup>* gene was amplified from a wild-type (CHP429) gDNA template by PCR using the Atg3oef and Atg3oer oligonucleotide primers (see Table 2.2), which also introduced a *NdeI* restriction site before the START codon and a *BamHI* restriction site after the STOP codon, respectively. After digestion with *NdeI* and *BamHI* restriction enzymes, the PCR product was ligated with *NdeI/BamHI*-digested pREP41 vector. To overexpress *atg3<sup>+</sup>*, pREP41-*atg3* was introduced into *S. pombe*, and positive transformants were selected by growth on media lacking leucine.



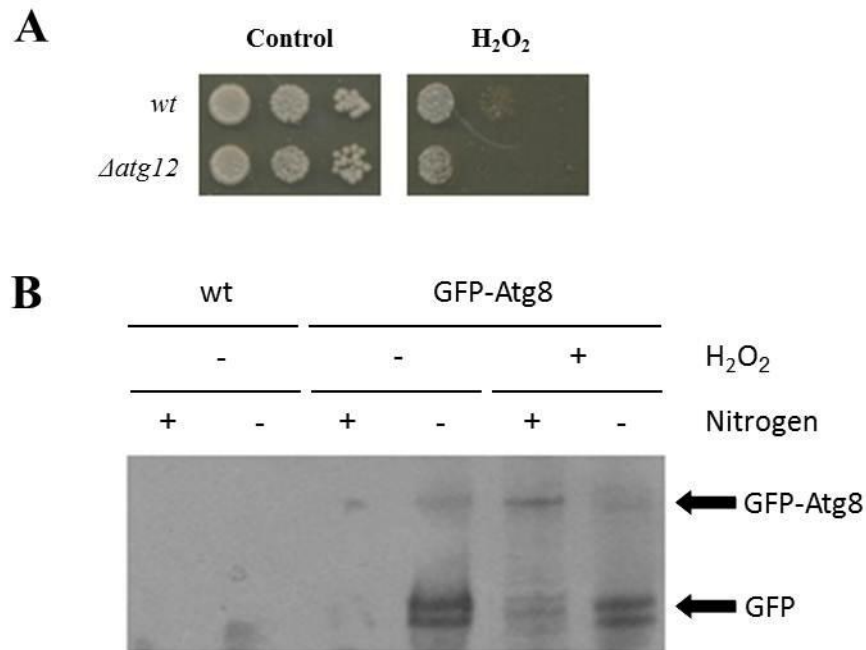
**Figure 5.10. Oxidative stress did not induce the formation of HMW complexes involving Atg7 in cells expressing *atg3*<sup>+</sup> from pREP41-*atg3*.**

Atg7Pk (MF01) cells and Atg7Pk cells containing pREP41-*atg3* (MF54) cells were treated with the indicated concentrations of H<sub>2</sub>O<sub>2</sub> for 20 minutes. Extracts were prepared by TCA lysis, and analysed by western blotting under non-reducing conditions, using anti-Pk antibodies. An extract from wild-type (wt; CHP428) cells expressing untagged protein was also analysed, as a control. Atg7Pk is labelled, and a slower mobility form of Atg7Pk is indicated by an asterisk. The position on the blot including proteins of the M<sub>r</sub> expected for E1-E2 complexes is indicated by a red line. This experiment was repeated twice with the same result.

generation. Therefore, it was possible that autophagy contributes to oxidative stress resistance in *S. pombe*, as in other organisms. Hence, to determine whether autophagy is required for resistance to H<sub>2</sub>O<sub>2</sub>, the H<sub>2</sub>O<sub>2</sub>-sensitivity of cells lacking Atg12 was assessed. Interestingly,  $\Delta atg12$  cells did not display increased sensitivity to H<sub>2</sub>O<sub>2</sub> (**Fig. 5.11A**), suggesting that autophagy is not required for resistance to H<sub>2</sub>O<sub>2</sub> in *S. pombe*. In light of this result, it was possible that autophagy is not induced by oxidative stress in *S. pombe*, in contrast to higher organisms. To investigate this possibility, we next investigated whether oxidative stress affects GFP-Atg8 hydrolysis in *S. pombe*. GFP release was not detectable following treatment of GFP-Atg8 cells with a range of concentrations of H<sub>2</sub>O<sub>2</sub> and diamide (data not shown). However, it was possible that oxidative stress could be involved in the regulation of autophagic activity following induction of autophagy by nitrogen starvation. Hence, GFP-Atg8 cells were incubated in nitrogen-free medium, and simultaneously treated with H<sub>2</sub>O<sub>2</sub>. GFP release was not significantly altered in H<sub>2</sub>O<sub>2</sub>-treated cells compared with untreated cells, indicating that H<sub>2</sub>O<sub>2</sub> levels do not increase autophagic activity in *S. pombe* (**Fig. 5.11B**).

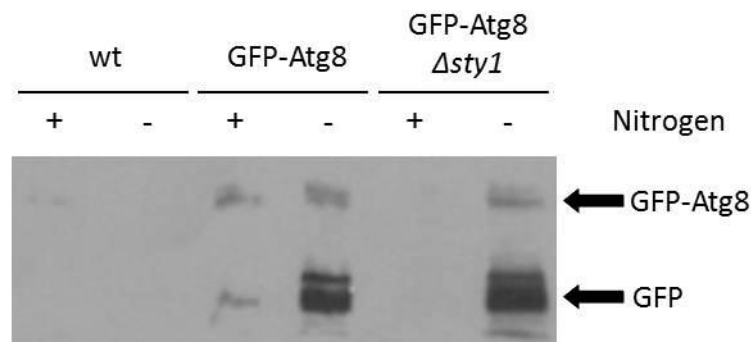
#### **5.2.6. Sty1 is not involved in the regulation of autophagy.**

Interestingly, previous investigations suggest that the SAPK Sty1 and the transcription factor Atf1 may be involved in autophagy regulation. Sty1 and Atf1 are required for entry into quiescence (Sajiki et al., 2009), Sty1 is activated by nitrogen starvation, and Atf1 regulates gene expression during nitrogen starvation (Takeda et al., 1995). Moreover, TOR homologues become inactivated during starvation, leading to increased Sty1 activation via downregulation of the Pyp2 phosphatase (Petersen and Nurse, 2007). Decreased TOR activity has long been associated with induction of autophagy, and it is possible that Sty1 plays a role in this. Finally, the mammalian SAPK JNK promotes autophagy by phosphorylating Bcl-2 (Geeraert et al., 2010; Pattingre et al., 2005; Wei et al., 2008), although it is unclear whether SAPKs can also regulate autophagy in lower organisms. To investigate the possible involvement of Sty1 in autophagy regulation, a GFP-Atg8  $\Delta sty1$  strain lacking auxotrophic markers (MF81) was constructed by genetic cross between the AD22 and JT268 strains. GFP-Atg8  $\Delta sty1$  cells were then incubated in nitrogen-free medium for 20 hours to induce autophagy, and GFP release from the GFP-Atg8 fusion protein was examined by western blotting. Loss of Sty1 did not



**Figure 5.11. Autophagy is not required for resistance to  $H_2O_2$ , and overall autophagy is not affected by  $H_2O_2$  in *S. pombe*.**

**A.** To investigate whether autophagy is required for resistance to  $H_2O_2$ , ten-fold serial dilutions of mid-log phase growing wild-type (wt; CHP429) and  $\Delta atg12$  (MF13) cells were spotted onto YE5S plates containing the indicated concentrations of  $H_2O_2$  and incubated at 30°C for three days. **B.** GFP-Atg8 (JT268) and wild-type (wt; SW576) cells lacking auxotrophic markers were incubated in EMM or EMM-N for 20 hours at 30°C. To examine the effect of  $H_2O_2$  on GFP release, EMM and EMM-N included 1mM  $H_2O_2$ , as indicated. These results are representative of three independent experiments.



**Figure 5.12. The Sty1 pathway is not required for nitrogen-starvation-induced autophagy.**

GFP-Atg8 (JT268), GFP-Atg8  $\Delta sty1$  (MF81) and wild-type (wt; SW576) cells lacking auxotrophic markers were incubated in EMM or EMM-N for 20 hours at 30°C. Cellular extracts were prepared, and GFP release from the GFP-Atg8 fusion protein was investigated by western blotting using anti-GFP antibodies. This experiment was repeated and gave the same result.

appear to affect GFP release (**Fig. 5.12**), suggesting that the Sty1 pathway is not required for nitrogen starvation-induced autophagy. However, it remains possible that Sty1 influences the rate of autophagy, and the levels of free GFP at different time-points could be investigated in the future.

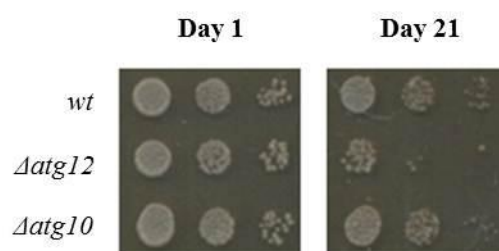
### **5.3. SpAtg10 has roles in oxidative stress resistance and cell cycle progression, but not in autophagy.**

Although a fission yeast homologue of Atg10 cannot be easily identified using the amino acid sequence of the whole protein, a potential homologue has been identified due to the presence of a  $\Psi$ HPC motif commonly found in autophagic E2s, and has been named SpAtg10 (see Section 5.2.2). However, initial analysis suggests that SpAtg10 does not function in autophagy. In this section, the functions of SpAtg10 will be investigated further.

#### **5.3.1. SpAtg10 is not required for autophagy.**

Autophagy is required for the long-term survival of *S. pombe* during nitrogen starvation, likely by providing an alternative nitrogen source via the degradation of cellular components (Kohda et al., 2007). Although the GFP release assay allows autophagic activity to be monitored, this assay does not provide an insight into how dysfunctional autophagy affects long-term survival. Hence, to investigate the roles of Atg12 and SpAtg10 in long-term survival, wild-type,  $\Delta atg12$  and  $\Delta atg10$  cells lacking auxotrophic markers were grown in nitrogen-free medium for up to three weeks. Cells were spotted onto solid YE5S medium at regular intervals. Although  $\Delta atg12$  and  $\Delta atg10$  cells showed similar viability to wild-type cells up to two weeks after being transferred to nitrogen-free medium (data not shown),  $\Delta atg12$  viability was significantly decreased thereafter (**Fig. 5.13**). A similar loss of viability was reported for  $\Delta atg1$ ,  $\Delta atg8$  and





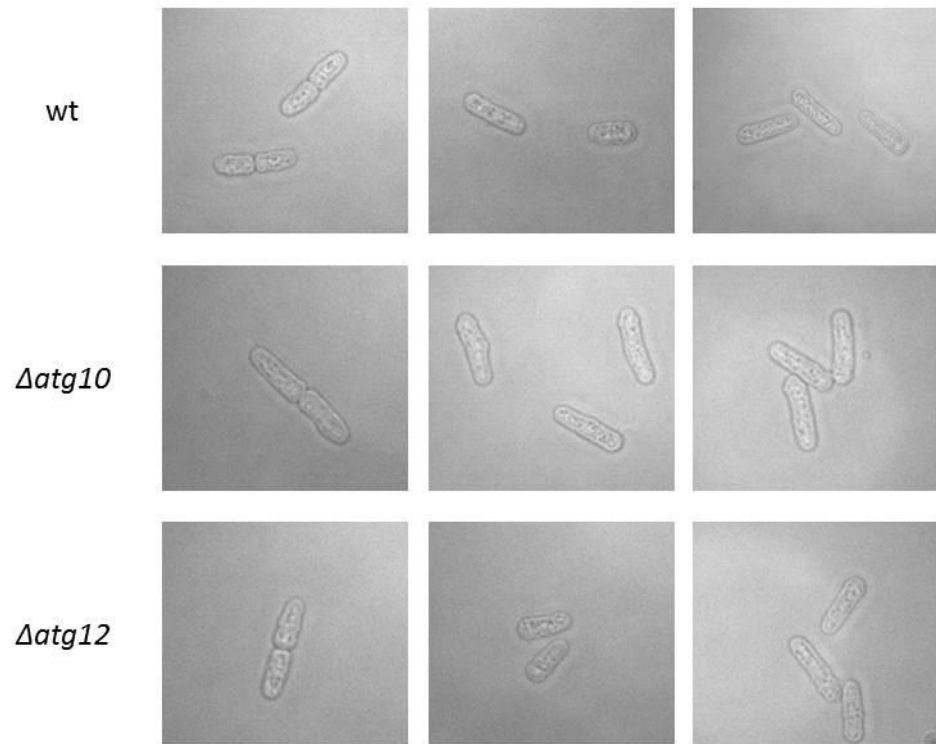
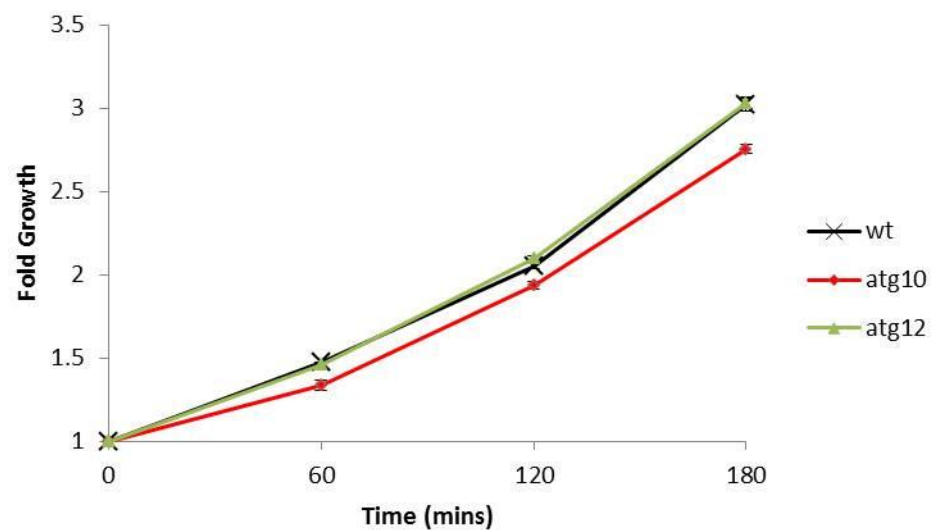
**Figure 5.13. Atg12, but not SpAtg10, is required for the viability of *S. pombe* cells during long-term nitrogen starvation.**

To determine whether Atg12 and/or SpAtg10 are required for long-term survival during nitrogen starvation,  $\Delta atg12$  (MF13)  $\Delta atg10$  (MF15) and wild-type (wt; SW576) cells lacking auxotrophic markers were incubated at 30°C in EMM-N medium for three weeks. Growth medium was removed and replaced with fresh medium every three days. Cells were spotted onto YE5S plates after the number of days indicated. YE5S plates were incubated at 30°C for 3 days. This experiment was repeated and yielded a similar result.

*Δatg13* cells following weeks of nitrogen starvation (Kohda et al., 2007), demonstrating that autophagy is required for the long-term viability of fission yeast cells during nitrogen starvation, and confirming the earlier finding that Atg12 is required for autophagy. In contrast, *Δatg10* cells did not show loss of viability, even after growth in nitrogen-free medium for three weeks (**Fig. 5.13**). This again suggests that SpAtg10 is not involved in autophagy, and does not facilitate Atg12 conjugation.

### **5.3.2. SpAtg10 has roles in oxidative stress resistance and cell cycle regulation.**

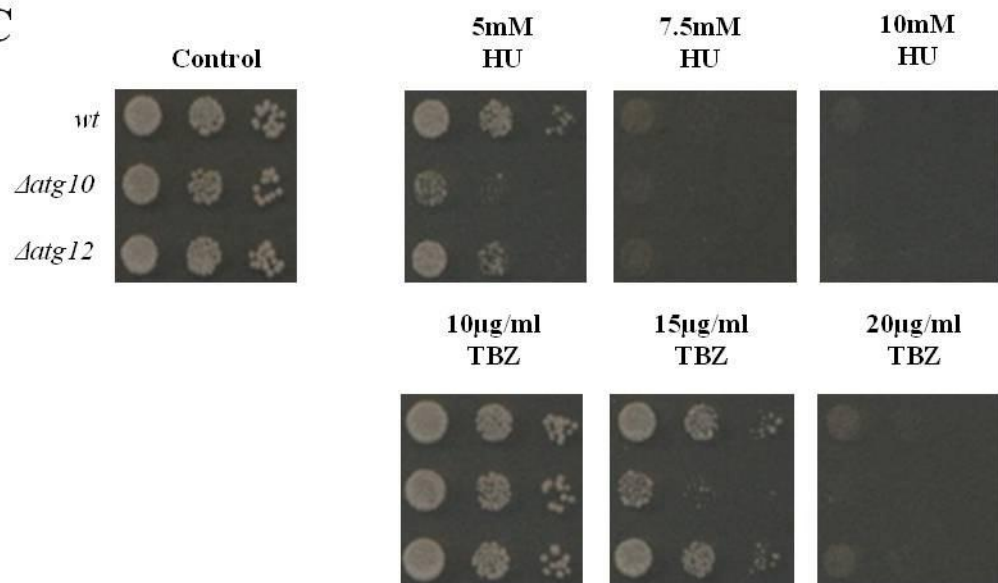
Having established that SpAtg10 is not involved in autophagy, the possibility that the enzyme has roles in controlling other cellular processes was investigated. Firstly, *Δatg10* cells were examined by light microscopy. These mutant cells were generally longer than wild-type cells, and showed slight differences in cellular morphology, including kinks and small bulges (**Fig. 5.14A**). Importantly, this was not the case for *Δatg12* cells, which were indistinguishable from wild-type cells. In addition, *Δatg10* cells grew slightly more slowly than wild-type or *Δatg12* cells at mid-log-phase (**Fig. 5.14B**). Furthermore, when analysing cell growth, additional data from the cell counter indicated that wild-type and *Δatg12* cells have an average diameter of  $\sim 5.9\mu\text{m}$  and an approximate volume of  $\sim 130\text{fl}$  when growing at mid-log-phase. In contrast, *Δatg10* cells were  $\sim 7\mu\text{m}$  in length and had an average volume of over 200fl. These data support the initial observation that *Δatg10* cells are larger than wild-type and *Δatg12* cells, and suggest that SpAtg10 may have roles in controlling cell morphology and/or the cell cycle. To investigate this possibility, *Δatg10* cells were exposed to agents affecting the cell cycle, including the ribonucleotide reductase inhibitor HU and the spindle-depolymerising agent TBZ. In contrast to *Δatg12* cells, *Δatg10* cells displayed increased sensitivity to both HU and TBZ (**Fig. 5.14C**). This again suggests that SpAtg10 (but not Atg12) may be involved in the control of the cell cycle in *S. pombe*. To determine whether SpAtg10 has a role in oxidative stress responses, *Δatg10* cells were also grown on media containing oxidative stress agents. Unexpectedly, *Δatg10* cells were more resistant to  $\text{H}_2\text{O}_2$  and diamide than wild-type cells (**Fig. 5.14D**). These results indicate that SpAtg10 may also play a role in controlling oxidative stress resistance in fission yeast. Interestingly, *Δatg12* cells are also resistant to diamide, but not as resistant as *Δatg10* cells. Taken together, these data suggest that SpAtg10

**A****B**

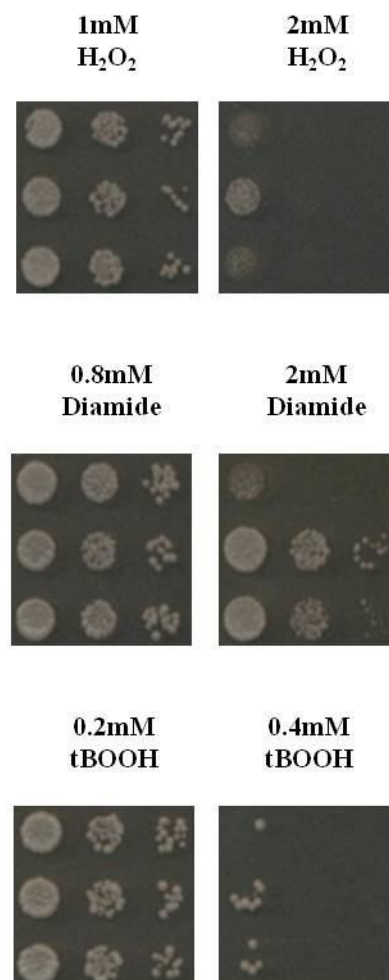
**Figure 5.14.  $\Delta atg10$  cells display minor changes in growth rate and cellular morphology, and increased sensitivity to the microtubule-depolymerising agent TBZ.**

**A.** Mid-log-phase, growing  $\Delta atg10$  (MF15),  $\Delta atg12$  (MF13) and wild-type (wt; SW576) cells were examined by light microscopy. **B.** The number of cells in mid-log-phase  $\Delta atg10$ ,  $\Delta atg12$  and wild-type (wt) cultures were counted at 1 hour intervals. Fold growth was calculated relative to the number of cells at time 0. The data points represent mean cell numbers  $\pm$  standard error, calculated from data recorded in three independent experiments. **C, D.** To investigate sensitivity to cell cycle stresses, ten-fold serial dilutions of mid-log phase growing  $\Delta atg10$ ,  $\Delta atg12$  and wild-type (wt) cells were spotted onto YE5S plates containing the indicated concentrations of (C) HU and TBZ. To investigate sensitivity to oxidative stresses, cells were spotted onto YE5S plates containing the indicated concentrations of (D)  $H_2O_2$ , diamide and *t*-BOOH. Plates were incubated at 30°C for three days. These experiments were repeated twice with the same results.

**C**



**D**

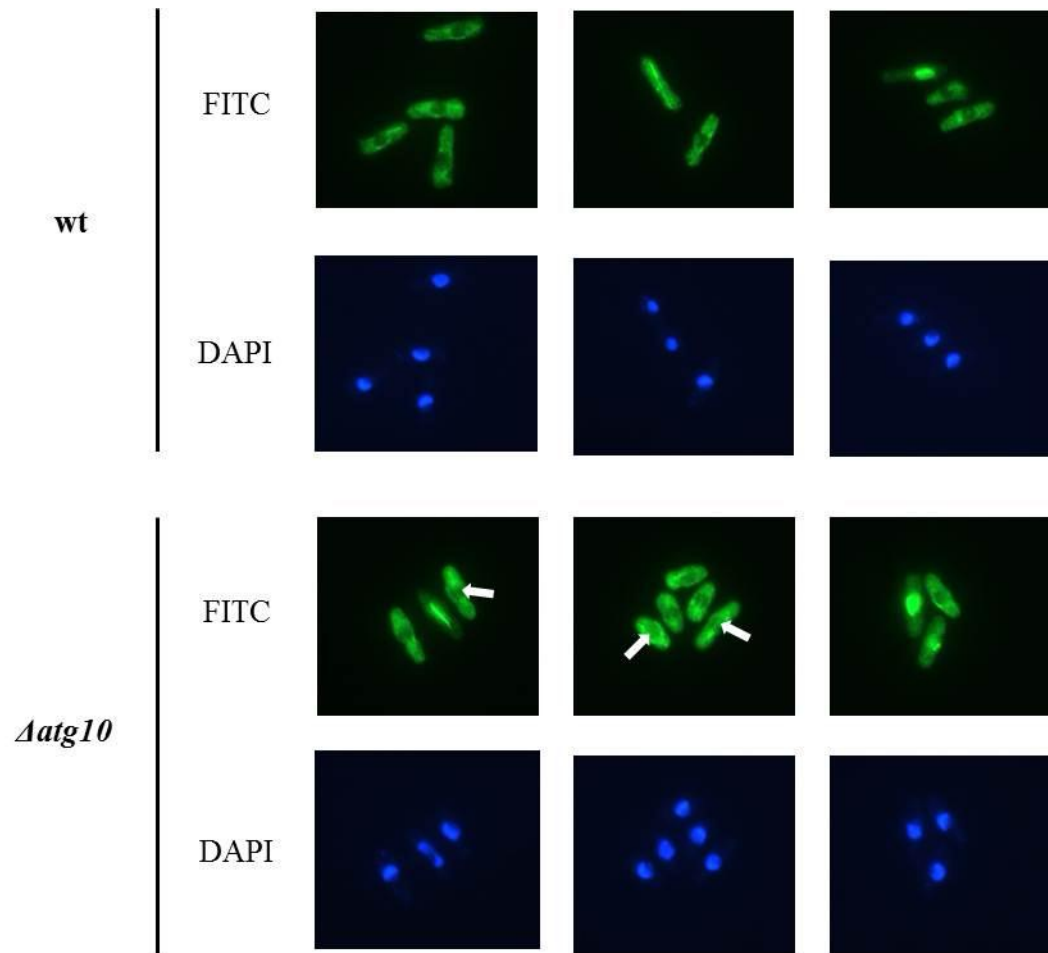


functions independently of autophagic Ubl conjugation to regulate cell cycle progression and oxidative stress resistance.

Given that  $\Delta atg10$  cells display increased sensitivity to TBZ, it was possible that SpAtg10 plays a role in the organisation of microtubules and/or the mitotic spindle. Hence, the appearance of microtubules in growing  $\Delta atg10$  cells was analysed by indirect immunofluorescence. Microtubule organisation in  $\Delta atg10$  cells did not differ significantly from wild-type cells (**Fig. 5.15**), suggesting that the formation of microtubule fibres is not prevented by the absence of SpAtg10. However, there were subtle differences in the appearance of microtubules in  $\Delta atg10$  cells. In wild-type cells, microtubules arrange in bundles around the edge of the cell, and appear to be fully excluded from the nucleus until the breakdown of the nuclear envelope during mitosis (**Fig. 5.15**). In  $\Delta atg10$  cells, there is often a fluorescent signal across the nucleus prior to breakdown of the nuclear envelope (**Fig. 5.16**), suggesting that microtubules may penetrate the nucleus or become concentrated in a manner not seen in wild-type cells. Although the relationship between SpAtg10 and microtubule organisation requires further investigation, it is possible that SpAtg10 affects microtubule organisation in fission yeast.

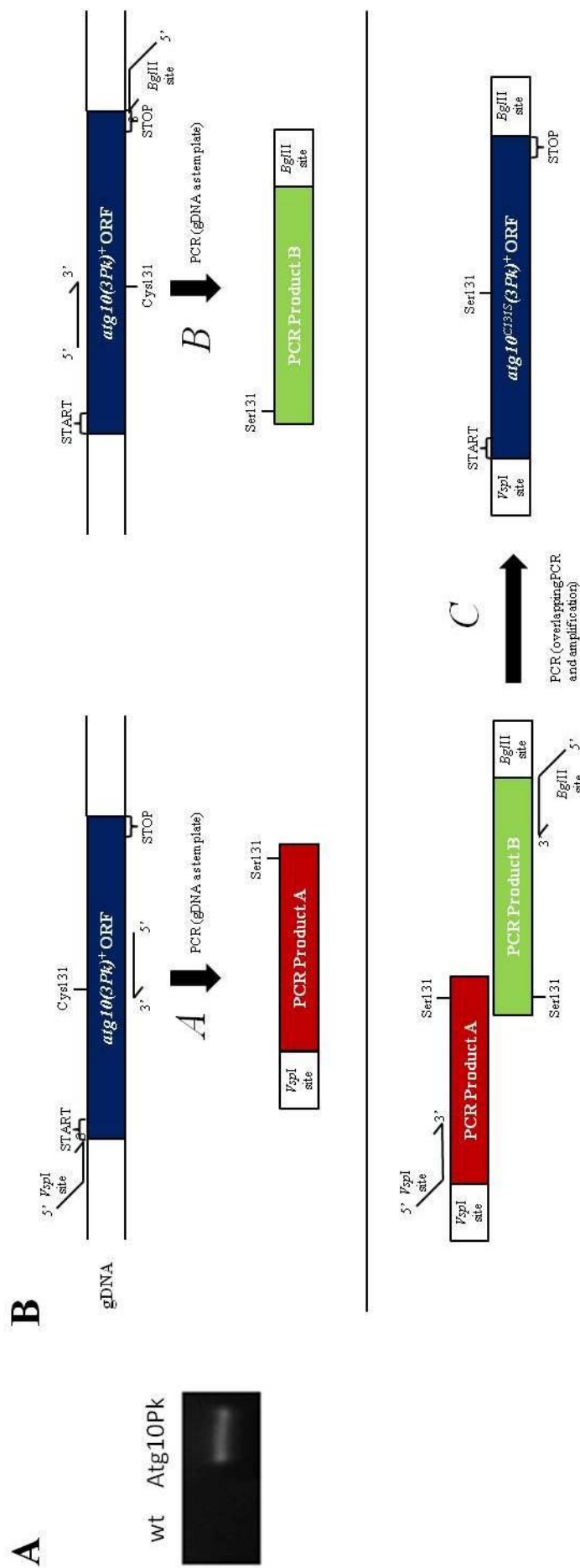
### **5.3.3. The putative catalytic cysteine is an important determinant of SpAtg10 stability.**

It was possible that SpAtg10 may facilitate the conjugation of a ubiquitin/Ubl other than Atg12, if the enzyme does not function in autophagy. Importantly, if the catalytic cysteine of an E1 or E2 is replaced by a serine, an irreversible ester bond is formed between the enzyme and its cognate Ubl. Thus, the Ubl is bound to the enzyme, but cannot be transferred to downstream substrates. Hence, E1-Ubl or E2-Ubl ester complexes should be readily detectable in cellular extracts. Cys-Ser substitutions can therefore help to identify the Ubl bound by a particular enzyme. To attempt to identify the Ubl bound by SpAtg10, a SpAtg10<sup>C131S</sup> mutant protein tagged with Pk epitopes was expressed. First, the integration tagging plasmid pRIP42PkC-*atg10* was constructed (see Section 2.1.5.1), linearised, and introduced into wild-type cells. Integration at the



**Figure 5.15. *Δatg10* cells show morphological differences to wild-type cells, but there are no obvious differences in mitotic spindle appearance when exposed to TBZ.**

To examine the mitotic spindle, wild-type (wt; CHP429) and *Δatg10* (MF78) cells were fixed with 3.7% paraformaldehyde. Cells were treated with 15μg/ml TBZ for 3 hours prior to fixation, as indicated. Spindles were visualised by indirect immunofluorescence using mouse anti-TAT1 antibodies and Alexa Fluor®-conjugated anti-mouse antibodies. Nuclei were visualised by DAPI staining. A fluorescent signal across the nuclei of *Δatg10* cells is indicated by arrows. This experiment was repeated and gave the same result.



**Figure 5.16. SpAtg10<sup>Cl31S</sup>Pk was generated by overlapping PCR.**

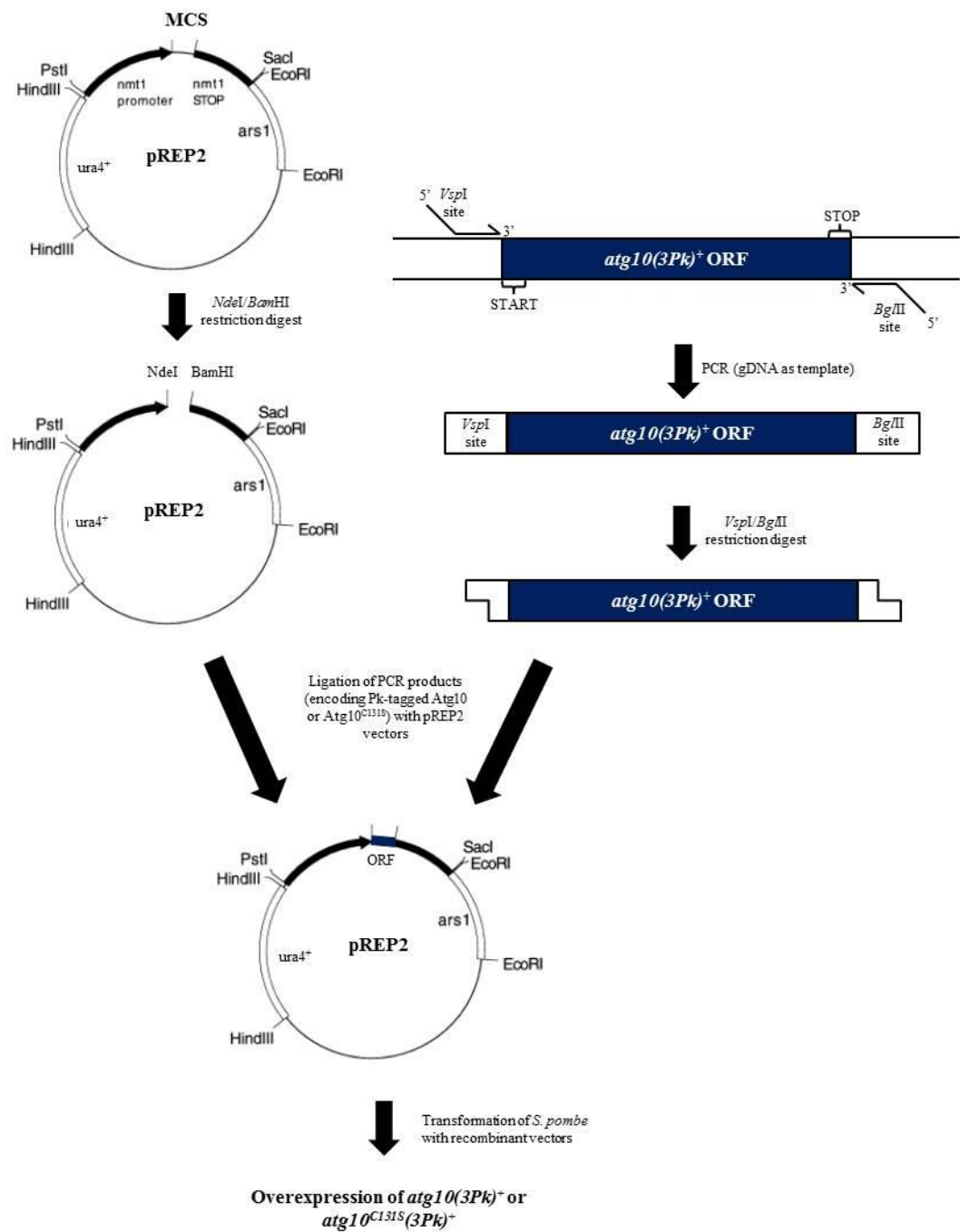
A. To confirm plasmid integration, gDNA was extracted from wild-type (wt; CHP428) cells and from cells transformed with linearised pRIP42PkC-*atg10* to tag SpAtg10 with Pk epitopes (Atg10Pk; MF11). Integration at the correct locus was confirmed by PCR using the forward primer Atg10chkf, and the reverse primer mntend (see Table 2.2), and using gDNA extracts as templates. PCR products were analysed by agarose gel electrophoresis, and a product of ~500bp was taken as confirmation of plasmid integration. B. PCR reaction A was performed using the oligonucleotide primers Atg10oef and Atg10Cl31Supr, with gDNA extracted from Atg10Pk (MF11) cells as a template. PCR reaction B was performed using the oligonucleotide primers Atg10Cl31Sdnf and Atg10Pkoe, with gDNA extracted from SpAtg10Pk (MF11) as a template. Equimolar amounts of PCR Products A and B were mixed, and ten cycles of PCR were performed to begin PCR reaction C. The oligonucleotide primers Atg10oef and Atg10Pkoe were then added, before restarting the PCR reaction to amplify the product generated by overlapping PCR. The final PCR product encodes SpAtg10<sup>Cl31S</sup>Pk, flanked by *VspI* and *BglII* restriction sites. Oligonucleotide primer sequences are listed in Table 2.2.



*atg10*<sup>+</sup> locus was confirmed by PCR (**Fig. 5.16A**). Next, an overlapping PCR-based technique was used for site-directed mutagenesis (Ho et al., 1989) where the putative catalytic cysteine residue of SpAtg10Pk was mutated to a serine (SpAtg10<sup>C131S</sup>Pk) (**Fig. 5.16B**). PCR products encoding Pk epitope-tagged SpAtg10 and SpAtg10<sup>C131S</sup> were used to construct expression plasmids (**Fig. 5.17**). These plasmids were designated pREP2-*atg10*(3Pk) and pREP41-*atg10*<sup>C131S</sup>(3Pk) (see Section 2.1.5.2), and were introduced into cells to overexpress SpAtg10Pk and SpAtg10<sup>C131S</sup>Pk. When pREP2-*atg10*(3Pk) was introduced into cells, SpAtg10Pk was barely detectable in cellular extracts. However, when pREP41-*atg10*<sup>C131S</sup>(3Pk) was used, SpAtg10<sup>C131S</sup>Pk was readily detectable as a doublet (**Fig. 5.18**). In both cases, slower mobility forms of SpAtg10Pk were detected, which may suggest that the protein is post-translationally modified. Taken together, these data suggest that the putative catalytic cysteine residue is an important determinant of SpAtg10 stability. Furthermore, SpAtg10 may be regulated by post-translational modification, and the shift in mobility of the protein indicates that it may be modified by ubiquitination or Ubl conjugation.

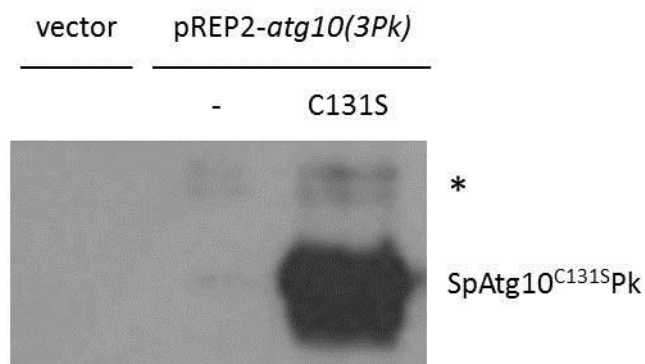
Although the molecular function of SpAtg10 remains unknown, this protein appears to play a role in promoting cell growth in sub-optimal growth conditions, and/or in the regulation of the spindle checkpoint. Furthermore, loss of SpAtg10 appears to increase resistance to oxidative stress agents such as diamide. Interestingly, attempts to cross  $\Delta$ *atg10* with other strains were unsuccessful (data not shown), indicating that SpAtg10 may also have important roles in meiosis. These results could have wider implications for the study of autophagic Ubl conjugation. Firstly, Atg12 conjugation to Atg5 may be facilitated by an E2 other than Atg10, in some organisms. Indeed, recent evidence suggests that Atg3 can function as an E2 to facilitate Atg12 conjugation in mammalian cells, and that mammalian Atg12 can be conjugated to many proteins other than Atg5 (Radoshevich et al., 2010). Furthermore, Atg10 does not appear to be essential for Atg12-Atg5 formation in mammalian cells (Mizushima et al., 2002; Nemoto et al., 2003). This could also be the case in *S. pombe*. Secondly, it is possible that Atg10 homologues in other organisms may also function in cell cycle control. Finally, identifying E2s functioning in autophagic Ubl conjugation in eukaryotes may be more complicated than previously thought, as SpAtg10 includes a  $\Psi$ HPC motif characteristic of autophagic E2s, yet does not appear to function in autophagy.





**Figure 5.17.** Both *atg10(3Pk)<sup>+</sup>* and *atg10<sup>C131S</sup>(3Pk)<sup>+</sup>* were overexpressed using expression vectors.

ORFs were amplified from Atg10Pk and Atg10<sup>C131S</sup>Pk DNA templates by PCR, using the Atg10oef and Atg10Pkoer oligonucleotide primers (see Table 2.2), which introduced a *VspI* restriction site before the START codon and a *BglII* restriction site after the STOP codon, respectively. After digestion with *VspI* and *BglII* restriction enzymes, PCR products were ligated with *NdeI/BamHI*-digested vectors. Recombinant vectors were introduced into *S. pombe*, and positive transformants were selected by growth on media lacking uracil.



**Figure 5.18. Mutation of the catalytic cysteine increases the stability of SpAtg10.**

Cellular extracts were prepared from cells containing recombinant vectors overexpressing *atg10(3Pk)*<sup>+</sup> (MF51) and *atg10<sup>C131S</sup>(3Pk)*<sup>+</sup> (MF55), and from wild-type (vector; CHP429) cells. Extracts were analysed by western blotting, using anti-Pk antibodies. SpAtg10<sup>C131S</sup>Pk is labelled. Possible modification of SpAtg10 is indicated by an asterisk. This experiment was repeated and gave the same result.

## 5.4. Discussion

In this chapter, the control of autophagy by oxidative stress and autophagic Ubl conjugation was investigated in *S. pombe*, using a novel GFP release assay. As in other organisms, fission yeast autophagy is induced by nitrogen starvation. However, while oxidative stress has been repeatedly linked to autophagy induction in higher eukaryotes, fission yeast autophagy was not increased in response to elevated concentrations of ROS, suggesting that autophagic activity can be regulated by conserved and organism-specific mechanisms. In agreement with this idea, Atg4 does not appear to be redox-regulated in *S. pombe*, unlike in mammalian cells. However, Atg4 appears to be post-translationally modified in *S. pombe*, and the levels of this modification are influenced by nitrogen starvation, indicating that post-translational modification of Atg4 may be important for the induction of autophagy during nitrogen starvation. Furthermore, in contrast to other enzymes functioning in ubiquitin/Ubl conjugation, Atg7 does not form HMW disulfide complexes following exposure to H<sub>2</sub>O<sub>2</sub>. Finally, a protein predicted to be the fission yeast homologue of Atg10 is dispensable for autophagy, and instead functions in oxidative stress responses and cell cycle control. This finding may have implications for the study of autophagic Ubl conjugation in other organisms.

To investigate the roles and regulation of autophagic Ubl conjugation pathways, and how this influences autophagy as a whole, an assay for autophagy was required. Although previous investigations have used protein degradation as a measure of autophagic activity in *S. pombe* (Kohda et al., 2007), proteins can also be broken down via the ubiquitin-proteasome system, meaning that protein degradation may not reflect autophagic activity. Therefore, GFP release from a GFP-Atg8 fusion protein was monitored using western blotting. GFP-Atg8 breakdown was increased following hours of nitrogen starvation. Critically, GFP release was largely prevented when *atg12*<sup>+</sup> was mutated, or when cells were grown in the presence of the vacuolar protease inhibitor PMSF. During the course of this project, another group also monitored GFP release from a GFP-Atg8 fusion protein to investigate autophagic activity in *S. pombe* (Mukaiyama et al., 2009). Similarly, they validated this assay using *atg* gene mutants, and also demonstrated that Atg12 is required for autophagy during nitrogen starvation.

GFP-Atg8 breakdown was also inhibited in many other strains, including  $\Delta atg3$ ,  $\Delta atg4$ ,  $\Delta atg5$ ,  $\Delta atg7$  and  $\Delta atg8$  (Mukaiyama et al., 2009). This confirms that autophagic Ubl conjugation is required for autophagy in fission yeast, as in other organisms. In the future, this GFP release assay can be used to monitor the effects of various stimuli on autophagic activity, and to investigate whether different proteins are involved in autophagy in fission yeast. Notably, the results of these GFP release assays support a previous report that autophagy is induced much more slowly by nitrogen starvation in *S. pombe* than in *S. cerevisiae* (Kohda et al., 2007). Although the reason for this is unclear, it has been suggested that an alternative nitrogen source may be available in *S. pombe*, and that autophagy may only be significantly upregulated following depletion of this nitrogen source (Mukaiyama et al., 2010).

Having established that the level of GFP release reflects autophagic activity in *S. pombe*, this assay was used to investigate how fission yeast autophagy is affected by oxidative stress. Interestingly, autophagy was not induced by  $H_2O_2$ , and elevated concentrations of  $H_2O_2$  did not affect autophagy induction during nitrogen starvation. In contrast, many studies have linked oxidative stress to increased autophagic activity in mammalian cells (Azad et al., 2009; Bensaad et al., 2009; Chen et al., 2007, 2008b; Huang et al., 2009). Additionally, Sty1 does not appear to regulate autophagy in *S. pombe*, whilst the mammalian SAPK JNK promotes autophagy via phosphorylation of Bcl-2 (Geeraert et al., 2010; Pattingre et al., 2005; Wei et al., 2008). Taken together, these results suggest that the induction of autophagy may be a species-specific response to oxidative stress in some eukaryotes, whereas increased autophagic activity could be a conserved response to other stimuli, such as nitrogen starvation. Interestingly, recent data has shown that autophagy is involved in the removal of dysfunctional mitochondria during G0 phase in *S. pombe*, and that this may prevent excessive ROS generation (Takeda et al., 2010). Hence, although autophagy does not appear to be required for resistance to oxidative stress in growing cells, autophagy may have dual roles in providing a nitrogen source and protecting against oxidative stress to maintain viability during quiescence.

In mammalian cells, Atg4 is inactivated by  $H_2O_2$  to promote retention of Atg8 on the growing autophagosome, thus increasing autophagic activity (Scherz-Shouval et al., 2007). However, it is important that a pool of Atg4 remains active elsewhere in the cell to process Atg8 from its inactive precursor into a form that can be activated by Atg7. It

is likely that localised ROS generation is important to Atg4 regulation in mammals. For example, increased ROS generation by dysfunctional mitochondria may cause local inactivation of Atg4, leading to the removal of these mitochondria by autophagy to protect cells from oxidative stress. However, much of the cellular pool of Atg4 would remain active, allowing Atg8 processing to continue (Scherz-Shouval and Elazar, 2007). Localised Atg4 regulation during nitrogen starvation is an attractive possibility in *S. pombe*, given that survival in G0 phase relies on the removal of mitochondria to prevent ROS generation (Takeda et al., 2010). Indeed, fission yeast Atg4 appears to be present in oxidised and reduced forms in cell extracts. However, the relative levels of Atg4<sup>ox</sup> and Atg4<sup>red</sup> are not affected by H<sub>2</sub>O<sub>2</sub>, indicating that Atg4 is not redox-regulated in *S. pombe*. As outlined in Section 1.2, while redox regulation of proteins is involved in a range of cellular process in higher organisms, redox regulation of proteins in lower organisms is largely geared towards promoting stress resistance. Hence, given that sensitivity tests indicate that autophagy is not required for resistance to a range of oxidative stress agents, it is perhaps unsurprising that Atg4 is not redox-regulated in *S. pombe*. However, a previously-unreported post-translational modification of Atg4 was observed, and the levels of this modification decreased during nitrogen starvation. This modification is likely to be phosphorylation, as the omission of phosphatase inhibitors from the lysis buffer decreases the levels of Atg4 modification. Previous investigations have linked decreases in post-translational modification of Atg proteins with the induction of autophagy. Indeed, deacetylation of Atg7, as well as Atg5 and Atg8, promotes autophagy in mammalian cells (Lee et al., 2008; Lee and Finkel, 2009; Salminen and Kaarniranta, 2009). It is possible that a similar loss of Atg4 modification regulates autophagy in *S. pombe*. The nature of this modification, and its effect on Atg4 function, require further characterisation. This possible phosphorylation of Atg4 may have implications for the control of autophagy both in *S. pombe* and in higher eukaryotes.

The possibility that Atg7 forms HMW disulfide complexes following cysteine oxidation was also investigated. In contrast to E1s and E2s functioning in other systems, Atg7 does not appear to form HMW disulfide complexes with other molecules following H<sub>2</sub>O<sub>2</sub> treatment. This was not unexpected, given that oxidative stress is associated with autophagy induction in eukaryotes, and HMW complex formation would be expected to inhibit autophagic Ubl conjugation. It could be significant that the structure of Atg7 is very different from the structures of other E1s. Atg7 forms homodimers via a C-

terminal domain (Komatsu et al., 2001), and interacts with Atg3 via the N-terminal, rather than a C-terminal Ubl fold (Taherbhoy et al., 2011). Hence, it is difficult to predict the environment of the catalytic cysteine residue of Atg7. Indeed, this is also the case for Atg3, which uses an insertion to the core E2 fold to facilitate its interaction with Atg7 in *S. cerevisiae* (Yamada et al., 2007). One of the advantages of these structures may be that cysteine oxidation is prevented, allowing autophagic Ubl conjugation to continue during oxidative stress.

As mentioned previously, another group have used a GFP release assay to monitor autophagic activity in *S. pombe*, and have confirmed that genes such as *atg3*<sup>+</sup>, *atg4*<sup>+</sup>, *atg5*<sup>+</sup>, *atg7*<sup>+</sup>, *atg8*<sup>+</sup> and *atg12*<sup>+</sup> are all required for autophagy (Mukaiyama et al., 2009). These genes all encode proteins predicted to function in autophagic Ubl conjugation, confirming that this is important for autophagy in *S. pombe*. However, the authors were unable to identify an Atg10 homologue in *S. pombe*. Here, a possible Atg10 homologue (SpAtg10) was identified, and its possible role in autophagy was investigated. Unexpectedly, GFP-Atg8 breakdown was unaffected when *atg10*<sup>+</sup> was mutated, and that  $\Delta$ *atg10* cells could enter G0 phase and survive long periods of nitrogen starvation. Moreover, the sensitivity phenotypes of  $\Delta$ *atg10* cells were different from those of  $\Delta$ *atg12* cells, and implicated SpAtg10 in oxidative stress responses and cell cycle control. Collectively, these results strongly suggest that SpAtg10 is not involved in autophagy in *S. pombe*. However, Atg12 and Atg5 are both essential for autophagy, and given that SpAtg10 was predicted to conjugate Atg12 to Atg5, it is now unclear which E2 facilitates Atg12 conjugation. Recent data has shown that Atg3 can facilitate Atg12 conjugation in mammals (Radoshevich et al., 2010), and Atg3 is required for optimal Atg12-Atg5 conjugate formation, even in the presence of Atg10 (Sou et al., 2008; Tanida et al., 2002). Hence, it is possible that Atg3 may function as a bifunctional E2, facilitating Atg12 conjugation to Atg5 and Atg8 conjugation to PE. This would be a unique system, as it would involve a bifunctional E1, Atg7, and a bifunctional E2, Atg3. Alternatively, Atg12 may be conjugated to Atg5 by an E2 other than Atg3 or Atg10. This may have significant implications for the study of autophagy in eukaryotes, and challenges the widely-held assumption that autophagic Ubl conjugation pathways are highly-conserved in eukaryotes. Finally, the autophagy-independent roles of SpAtg10 could suggest that Atg10 proteins have additional roles in other organisms, and may regulate processes such as cell cycle progression.



## Extra References

- Azad, M.B., Chen, Y., and Gibson, S.B. (2009). Regulation of autophagy by reactive oxygen species (ROS): implications for cancer progression and treatment. *Antioxidants & redox signaling* **11**, 777-790.
- Bensaad, K., Cheung, E.C., and Vousden, K.H. (2009). Modulation of intracellular ROS levels by TIGAR controls autophagy. *The EMBO journal* **28**, 3015-3026.
- Chen, D., Wilkinson, C.R., Watt, S., Penkett, C.J., Toone, W.M., Jones, N., and Bahler, J. (2008a). Multiple pathways differentially regulate global oxidative stress responses in fission yeast. *Molecular biology of the cell* **19**, 308-317.
- Chen, Y., McMillan-Ward, E., Kong, J., Israels, S.J., and Gibson, S.B. (2007). Mitochondrial electron-transport-chain inhibitors of complexes I and II induce autophagic cell death mediated by reactive oxygen species. *Journal of cell science* **120**, 4155-4166.
- Chen, Y., McMillan-Ward, E., Kong, J., Israels, S.J., and Gibson, S.B. (2008b). Oxidative stress induces autophagic cell death independent of apoptosis in transformed and cancer cells. *Cell death and differentiation* **15**, 171-182.
- Geeraert, C., Ratier, A., Pfisterer, S.G., Perdiz, D., Cantaloube, I., Rouault, A., Pattingre, S., Proikas-Cezanne, T., Codogno, P., and Pous, C. (2010). Starvation-induced hyperacetylation of tubulin is required for the stimulation of autophagy by nutrient deprivation. *The Journal of biological chemistry* **285**, 24184-24194.
- Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., and Pease, L.R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**, 51-59.
- Huang, J., Canadien, V., Lam, G.Y., Steinberg, B.E., Dinauer, M.C., Magalhaes, M.A., Glogauer, M., Grinstein, S., and Brumell, J.H. (2009). Activation of antibacterial autophagy by NADPH oxidases. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 6226-6231.
- Kohda, T.A., Tanaka, K., Konomi, M., Sato, M., Osumi, M., and Yamamoto, M. (2007). Fission yeast autophagy induced by nitrogen starvation generates a nitrogen source that drives adaptation processes. *Genes to cells : devoted to molecular & cellular mechanisms* **12**, 155-170.
- Komatsu, M., Tanida, I., Ueno, T., Ohsumi, M., Ohsumi, Y., and Kominami, E. (2001). The C-terminal region of an Apg7p/Cvt2p is required for homodimerization and is essential for its E1 activity and E1-E2 complex formation. *The Journal of biological chemistry* **276**, 9846-9854.
- Lee, I.H., Cao, L., Mostoslavsky, R., Lombard, D.B., Liu, J., Bruns, N.E., Tsokos, M., Alt, F.W., and Finkel, T. (2008). A role for the NAD-dependent deacetylase Sirt1 in the regulation of



autophagy. *Proceedings of the National Academy of Sciences of the United States of America* 105, 3374-3379.

Lee, I.H., and Finkel, T. (2009). Regulation of autophagy by the p300 acetyltransferase. *The Journal of biological chemistry* 284, 6322-6328.

Mikawa, T., Kanoh, J., and Ishikawa, F. (2010). Fission yeast Vps1 and Atg8 contribute to oxidative stress resistance. *Genes to cells : devoted to molecular & cellular mechanisms*.

Mizushima, N., Yoshimori, T., and Ohsumi, Y. (2002). Mouse Apg10 as an Apg12-conjugating enzyme: analysis by the conjugation-mediated yeast two-hybrid method. *FEBS letters* 532, 450-454.

Mukaiyama, H., Kajiwar, S., Hosomi, A., Giga-Hama, Y., Tanaka, N., Nakamura, T., and Takegawa, K. (2009). Autophagy-deficient *Schizosaccharomyces pombe* mutants undergo partial sporulation during nitrogen starvation. *Microbiology* 155, 3816-3826.

Mukaiyama, H., Nakase, M., Nakamura, T., Kakinuma, Y., and Takegawa, K. (2010). Autophagy in the fission yeast *Schizosaccharomyces pombe*. *FEBS letters* 584, 1327-1334.

Nemoto, T., Tanida, I., Tanida-Miyake, E., Minematsu-Ikeguchi, N., Yokota, M., Ohsumi, M., Ueno, T., and Kominami, E. (2003). The mouse APG10 homologue, an E2-like enzyme for Apg12p conjugation, facilitates MAP-LC3 modification. *The Journal of biological chemistry* 278, 39517-39526.

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X.H., Mizushima, N., Packer, M., Schneider, M.D., and Levine, B. (2005). Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* 122, 927-939.

Petersen, J., and Nurse, P. (2007). TOR signalling regulates mitotic commitment through the stress MAP kinase pathway and the Polo and Cdc2 kinases. *Nature cell biology* 9, 1263-1272.

Radoshevich, L., Murrow, L., Chen, N., Fernandez, E., Roy, S., Fung, C., and Debnath, J. (2010). ATG12 conjugation to ATG3 regulates mitochondrial homeostasis and cell death. *Cell* 142, 590-600.

Sajiki, K., Hatanaka, M., Nakamura, T., Takeda, K., Shimanuki, M., Yoshida, T., Hanyu, Y., Hayashi, T., Nakaseko, Y., and Yanagida, M. (2009). Genetic control of cellular quiescence in *S. pombe*. *Journal of cell science* 122, 1418-1429.

Salminen, A., and Kaarniranta, K. (2009). SIRT1: regulation of longevity via autophagy. *Cellular signalling* 21, 1356-1360.

Scherz-Shouval, R., and Elazar, Z. (2007). ROS, mitochondria and the regulation of autophagy. *Trends in cell biology* 17, 422-427.

Scherz-Shouval, R., Shvets, E., Fass, E., Shorer, H., Gil, L., and Elazar, Z. (2007). Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. *The EMBO journal* 26, 1749-1760.

Sou, Y.S., Waguri, S., Iwata, J., Ueno, T., Fujimura, T., Hara, T., Sawada, N., Yamada, A., Mizushima, N., Uchiyama, Y., *et al.* (2008). The Atg8 conjugation system is indispensable for proper development of autophagic isolation membranes in mice. *Molecular biology of the cell* 19, 4762-4775.

Su, S.S., Tanaka, Y., Samejima, I., Tanaka, K., and Yanagida, M. (1996). A nitrogen starvation-induced dormant G0 state in fission yeast: the establishment from uncommitted G1 state and its delay for return to proliferation. *Journal of cell science* 109 ( Pt 6), 1347-1357.

Taherbhoy, A.M., Tait, S.W., Kaiser, S.E., Williams, A.H., Deng, A., Nourse, A., Hammel, M., Kurinov, I., Rock, C.O., Green, D.R., *et al.* (2011). Atg8 transfer from Atg7 to Atg3: a distinctive E1-E2 architecture and mechanism in the autophagy pathway. *Molecular cell* 44, 451-461.

Takeda, K., Yoshida, T., Kikuchi, S., Nagao, K., Kokubu, A., Pluskal, T., Villar-Briones, A., Nakamura, T., and Yanagida, M. (2010). Synergistic roles of the proteasome and autophagy for mitochondrial maintenance and chronological lifespan in fission yeast. *Proceedings of the National Academy of Sciences of the United States of America* 107, 3540-3545.

Takeda, T., Toda, T., Kominami, K., Kohnosu, A., Yanagida, M., and Jones, N. (1995). *Schizosaccharomyces pombe atf1+* encodes a transcription factor required for sexual development and entry into stationary phase. *The EMBO journal* 14, 6193-6208.

Tanida, I., Tanida-Miyake, E., Komatsu, M., Ueno, T., and Kominami, E. (2002). Human Apg3p/Aut1p homologue is an authentic E2 enzyme for multiple substrates, GATE-16, GABARAP, and MAP-LC3, and facilitates the conjugation of hApg12p to hApg5p. *The Journal of biological chemistry* 277, 13739-13744.

Wei, Y., Pattingre, S., Sinha, S., Bassik, M., and Levine, B. (2008). JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. *Molecular cell* 30, 678-688.

Yamada, Y., Suzuki, N.N., Hanada, T., Ichimura, Y., Kumeta, H., Fujioka, Y., Ohsumi, Y., and Inagaki, F. (2007). The crystal structure of Atg3, an autophagy-related ubiquitin carrier protein (E2) enzyme that mediates Atg8 lipidation. *The Journal of biological chemistry* 282, 8036-8043.

## Chapter 6. Discussion

Ubiquitin/Ubl conjugation pathways are highly conserved in eukaryotes, and mediate post-translational modification of proteins to regulate their function, thus influencing many diverse cellular processes. Recently, SUMOylation has been shown to be inhibited during H<sub>2</sub>O<sub>2</sub> treatment in mammalian cells via the formation of an inhibitory disulfide complex involving Uba2 and Ubc9 (Bossis and Melchior, 2006). Similarly, Ubc12 can form HMW disulfide complexes following cysteine oxidation, resulting in inhibition of neddylation (Kumar et al., 2007). In addition, the Atg8-specific ULP, Atg4, can be inhibited via oxidation of a specific cysteine residue, thus promoting Atg8 retention on autophagosomal membranes and promoting autophagy (Scherz-Shouval et al., 2007). These data demonstrate that enzymes involved in ubiquitin/Ubl conjugation can be redox-regulated by H<sub>2</sub>O<sub>2</sub>. However, all of these studies were undertaken in mammalian cells, and it was therefore unclear whether ubiquitin/Ubl conjugation could also be redox-regulated in lower eukaryotes such as yeast. Moreover, how the redox regulation of ubiquitin/Ubl conjugation may impact upon oxidative stress responses in eukaryotes was not investigated, and it was unclear why only specific enzymes functioning in these pathways were redox-sensitive.

Recent research in our lab has demonstrated for the first time that specific enzymes facilitating ubiquitination can be redox-regulated in lower eukaryotes, and that this could be important for oxidative stress resistance. The E1, Uba1, and a specific E2, Cdc34, form inhibitory disulfide complexes following catalytic cysteine oxidation, leading to accumulation of the Cdk1 Sic1 and causing a transient cell cycle arrest in G1 phase (our unpublished data). This indicates that redox regulation of ubiquitin/Ubl conjugation is an important regulatory mechanism in both lower and higher eukaryotes. Furthermore, protein urmylation is upregulated during oxidative stress in both yeast and mammalian cells (Goehring et al., 2003a; Van der Veen et al., 2011). It is possible that a conserved redox regulatory mechanism influences urmylation in eukaryotes. Despite these exciting findings, it is notable that redox regulation of specific ubiquitin/Ubl conjugation pathways has never been shown to be conserved in eukaryotes.

In light of these results, the roles and regulation of ubiquitin/Ubl conjugation during oxidative stress were investigated in fission yeast, *S. pombe*. As in *S. cerevisiae*, a ubiquitin-specific E2, Ubc15, forms HMW disulfide complexes following exposure to oxidative stress agents. Ubc15 is also important for resistance to diamide, emphasising the importance of ubiquitin/Ubl conjugation in protecting cells against oxidative stress. Moreover, Ubc15 is a putative homologue of the redox-sensitive budding yeast E2, Cdc34, and may therefore represent the first example of conserved redox regulation of an E2 in eukaryotic cells. In contrast, the NEDD8-specific E2, Ubc12, and the aforementioned ULP, Atg4, do not appear to contain redox-sensitive cysteine residues within their structures, unlike their homologues in mammalian cells (Kumar et al., 2007; Scherz-Shouval et al., 2007), indicating that not all redox regulatory mechanisms are conserved in eukaryotes.

Interestingly, fission yeast Uba4 appears to contain redox-sensitive cysteine residues, suggesting that the enzyme can be redox-regulated. It is tempting to speculate that redox regulation of Uba4 is linked to the increased urmylation observed during oxidative stress in other organisms. Furthermore, the urmylation pathway has conserved roles in resistance to oxidative stress and heat, again showing that ubiquitin/Ubl conjugation pathways are important for stress resistance. In addition, the SAPK Sty1 appears to be regulated by urmylation. Although the underlying molecular mechanism of Sty1 regulation remains unclear, it is possible that SAPKs are also regulated by urmylation in other organisms, and that this could be important for stress resistance. Taken together, these findings advance the study of redox regulation of ubiquitin/Ubl conjugation in eukaryotes, and provide insights into how these pathways can influence stress resistance and various cellular processes.

### **6.1. Redox regulation of E1s and E2s to inhibit ubiquitin/Ubl conjugation, and the relevance for oxidative stress responses.**

As described in Chapter 1, the ubiquitination, SUMOylation and neddylation pathways involve E1s and E2s with similar overall structures and catalytic mechanisms, and some of these enzymes can be redox-regulated via oxidation of catalytic cysteine residues, resulting in the formation of inhibitory disulfide complexes. However, while redox regulation of ubiquitin/Ubl conjugation has been demonstrated in higher and lower eukaryotes, it was unclear whether any of these regulatory mechanisms are conserved. Furthermore, it was unclear why only specific E1s and E2s are redox-sensitive. Hence, the possible conservation of these redox regulatory mechanisms was investigated in *S. pombe*, and the reasons for redox-sensitivity of specific enzymes was considered.

In *S. cerevisiae*, Cdc34 is the only E2 that has been shown to form an inhibitory disulfide with Uba1 (our unpublished data). The formation of Uba1-Cdc34 disulfide complexes prevents Sic1 degradation during oxidative stress, causing cell cycle arrest in G1 phase of the cell cycle (our unpublished data). Cell cycle arrest may be an important feature of oxidative stress responses in budding yeast, as the transient accumulation of Sic1 may delay cell cycle progression to allow the repair of cellular damage and the restoration of redox homeostasis prior to DNA replication. In *S. pombe*, Ubc15 forms HMW disulfide complexes during oxidative stress, much like Cdc34. The  $M_r$  of these complexes suggests that they may be Uba1-Ubc15 disulfide complexes, although the identity of the components is unknown. Significantly, Ubc15 was predicted to be redox-sensitive due to similarity to Cdc34. Furthermore, both Cdc34 and Ubc15 lack acidic residues positioned near the catalytic cysteines of many E2s, and which are predicted to confer a high pKa to catalytic cysteines (Tolbert et al., 2005). However, while Cdc34 is known to facilitate the ubiquitination of Sic1 in *S. cerevisiae*, the substrates of Ubc15 are unknown. It is therefore unclear how redox-regulation of Ubc15 affects cellular processes in *S. pombe*. Given the similarity between budding yeast Cdc34 and fission yeast Ubc15, it is tempting to speculate that the fission yeast Sic1 orthologue, Rum1, could be a downstream substrate of Ubc15. However, while *CDC34* is an essential gene in *S. cerevisiae*, *ubc15*<sup>+</sup> is not essential in *S. pombe*, and  $\Delta$ *ubc15* cells do not display detectable defects in normal cell cycle progression. Furthermore, Ubc15 has been implicated in gene silencing in *S. pombe*, rather than cell cycle control (Nielsen et al., 2002). Thus, a different E2 may target Rum1, or multiple E2s may act redundantly to target Rum1 for degradation. The possible involvement of Ubc15 in Rum1 ubiquitination, and the effect of oxidative stress on Rum1 protein levels, requires further investigation. Indeed, to gain further insight into the possible involvement of Ubc15 in

oxidative stress responses, it will be important to identify proteins that are ubiquitinated in a Ubc15-dependent manner, and how this may be affected by oxidative stress.

Importantly, the redox-sensitivities of budding yeast Cdc34 and fission yeast Ubc15 differ in terms of the level of oxidative stress required to trigger disulfide complex formation. While Uba1-Cdc34 disulfide formation is triggered by mild oxidative stress, Ubc15 disulfide complexes were only observed when cells were exposed to high concentrations of H<sub>2</sub>O<sub>2</sub> or diamide (although it remains possible that Uba1-Ubc15 complexes are formed during milder oxidative stress, but are undetectable by western blotting, or that these complexes are only formed for a short time). It is possible that inactivation of Ubc15 is important for short-term survival during severe oxidative stress. Similarly, other enzymes may be inhibited by different levels of oxidative stress because their inhibition is important for survival at that particular level of stress. It is also possible that E1s and E2s are oxidised in specific locations within the cell in which H<sub>2</sub>O<sub>2</sub> concentrations are particularly high, as with the hyperoxidation and inactivation of Prx following RTK activation (see Section 1.2.2.4). For example, mitochondria are prominent sources of ROS, and H<sub>2</sub>O<sub>2</sub> concentrations are likely to be extremely high near the mitochondria, relative to other areas of the cell. In the future, it will be important to quantify H<sub>2</sub>O<sub>2</sub> concentrations at different positions in the cell, and relate this to the localisation and activity of enzymes such as Ubc15.

In addition to HMW disulfide formation, there was evidence that Ubc15 may be glutathionylated when exposed to milder concentrations of H<sub>2</sub>O<sub>2</sub>. Glutathionylation of catalytic cysteine residues may be another mechanism inhibiting ubiquitin/Ubl conjugation, and may also protect against cysteine hyperoxidation. Indeed, studies in mammalian cells have shown a decrease in E1-ubiquitin and E2-ubiquitin thioester formation during oxidative stress, and a concomitant increase in glutathionylation (Jahngen-Hodge et al., 1997). Therefore, glutathionylation of catalytic cysteines may inhibit ubiquitin/Ubl conjugation pathways during oxidative stress. However, it is unclear whether certain enzymes are more susceptible to glutathionylation than others. It is possible that certain E1s and E2s contain cysteines that are particularly sensitive to glutathionylation. This could be the case for Ubc15, and glutathionylation could inhibit Ubc15 activity, but also prevent intermolecular disulfide formation. Clearly, it is important to identify Ubc15 substrates, and monitor ubiquitination of these substrates under different conditions. For example, if substrate ubiquitination was decreased

during mild and severe oxidative stresses, it would suggest that glutathionylation and inhibitory disulfide formation perform similar inhibitory roles at different levels of stress.

Given that Uba1 transfers ubiquitin to multiple E2s, yet only appears to form inhibitory disulfide complexes with a limited number of them, it is possible that redox-regulation of ubiquitination, SUMOylation and neddylation is not controlled at the level of the E1. Instead, it may be the sensitivity of the E2 that drives disulfide formation. In support of this idea, Cdc34 and Ubc15 both lack a conserved aspartate residue that might be expected increase the pKa of the catalytic cysteine (Tolbert et al., 2005). However, while mammalian Ubc12 is particularly sensitive to oxidation (Kumar et al., 2007), this does not appear to be the case for fission yeast Ubc12, despite both the mammalian and yeast enzymes lacking the key aspartate residue near the catalytic cysteine. It is possible that the structure of Ubc12 is sufficiently different from those of Cdc34 and Ubc15 that different residues are positioned near the catalytic cysteine. By identifying more redox-sensitive E2s, and considering both their amino acid sequences and tertiary structures, it may be possible to predict which enzymes are more redox-sensitive than others. Alternatively, it would be interesting to investigate the effects of mutagenesis on redox-sensitivity of enzymes such as Ubc15, as this may identify key residues and motifs controlling redox-sensitivity.

In addition to the amino acid sequences and tertiary structures of specific enzymes, protein-protein interactions may also be important determinants of redox-sensitivity. It has been proposed that E1 thioester formation promotes the recruitment of an E2, due to changes in the structure of the E1 (Kerscher et al., 2006). The catalytic cysteine of the E2 becomes deprotonated during transthioesterification, and it is likely that a microenvironment generated during the E1-E2 interaction facilitates this deprotonation. Interestingly, Ubc9 is able to interact with Uba2 in the absence of SUMO (Wang et al., 2007), which does not appear to be the case for most E1s and E2s. It is possible that these interactions result in deprotonation of the Ubc9 catalytic cysteine without immediate transthioesterification, leading to oxidation and Uba2-Ubc9 disulfide formation. Furthermore, mammalian Ubc12 forms disulfide complexes with proteins other than Uba3 (Kumar et al., 2007). It is possible that the interactions between Ubc12 and specific proteins create microenvironments favouring deprotonation of the Ubc12 catalytic cysteine, leading to oxidation and disulfide formation to interacting proteins. It

is likely that a combination of E2 structure and amino acid sequence and microenvironments generated by protein-protein interactions all influence the redox-sensitivity of these enzymes.

## **6.2. Redox regulation promoting ubiquitin/Ubl conjugation, and relevance for oxidative stress responses.**

While ubiquitination, SUMOylation and neddylation can all be inhibited via oxidation of specific enzymes, it is also possible that redox regulation of enzyme activity can promote ubiquitin/Ubl conjugation. Previous investigations have shown that urmylation is induced by oxidative stress in *S. cerevisiae* (Goehring et al., 2003a) and in mammalian cells (Van der Veen et al., 2011), although how this is achieved remains unclear. Interestingly, fission yeast Uba4 contains redox-sensitive cysteine residues. It is possible that oxidation of Uba4 somehow promotes urmylation. As described in Chapter 1, Uba4 has a different structure to ‘canonical’ E1s such as Uba1, Uba2 and Uba3. In *S. cerevisiae*, the cysteine originally proposed to be the catalytic residue of Uba4 (Furukawa et al., 2000) does not bind Urm1 (Schmitz et al., 2008). Instead, a cysteine within the C-terminal RLD of Uba4 is now thought to be the catalytic residue, as mutation of this cysteine inhibits the enzyme, whereas mutation of the cysteine proposed to be the catalytic residue decreases Uba4 activity but does not inhibit the enzyme completely (Schmitz et al., 2008). Significantly, the catalytic mechanism of Uba4 may require deprotonation of non-catalytic cysteine residues (Schmitz et al., 2008). Cysteine deprotonation may render Uba4 sensitive to oxidation by H<sub>2</sub>O<sub>2</sub>, although how this may affect the function of Uba4 is unclear, and requires further investigation. However, it is possible that redox regulation of Uba4 at non-catalytic cysteines is linked to the induction of urmylation during oxidative stress.

In Chapter 4, the urmylation pathway was shown to be important for resistance to heat and oxidative stress agents such as diamide in *S. pombe*. Similarly, loss of the urmylation pathway results in increased sensitivity to heat and oxidative stress in *S.*



*cerevisiae* (Furukawa et al., 2000; Goehring et al., 2003a), demonstrating for the first time that urmylation has conserved roles in stress resistance in eukaryotes. It is tempting to speculate that the induction of protein urmylation observed during oxidative stress results in the increased urmylation of specific proteins that are important for stress resistance, and that Uba4 oxidation could be involved in this induction. However, an increase in protein urmylation was not detected when *S. pombe* cells were exposed to oxidative stress agents (although this could be due to technical issues). Moreover, evidence suggested that the role of Urm1 in promoting tRNA thiolation could be more important for resistance to diamide than the role of Urm1 in protein modification. How the urmylation of specific proteins may contribute to stress resistance remains an unanswered question. Additionally, urmylation appears to regulate the activity of Sty1, as dysfunctional urmylation results in increased Sty1 phosphorylation when cells are exposed to H<sub>2</sub>O<sub>2</sub>. This is the first time that urmylation has been linked to regulation of SAPKs in eukaryotes. This may have implications for the control of stress responses, and it would be interesting to investigate whether the highly-conserved urmylation pathway regulates SAPK activity in other organisms, and how this may influence stress resistance.

Regulation of the activity of DUBs and ULPs could also influence ubiquitin/Ubl conjugation to proteins. Indeed, Atg4 has been shown to be regulated in mammalian cells. Oxidation of a cysteine residue near the catalytic site of Atg4 inhibits the enzyme and promotes the retention of Atg8-PE conjugates on autophagosomal membranes (Scherz-Shouval et al., 2007). However, while redox regulation of mammalian Atg4 promotes autophagy, fission yeast Atg4 does not appear to be oxidised when cells are treated with H<sub>2</sub>O<sub>2</sub>, suggesting that Atg4 is not redox-regulated in all eukaryotes. It is perhaps significant that fission yeast Atg4 lacks the redox-sensitive cysteine residue identified within mammalian Atg4 (Scherz-Shouval et al., 2007). Furthermore, data presented in Chapter 5 suggests that autophagy is not important for oxidative stress resistance in *S. pombe*. While redox regulation is involved in controlling many diverse processes in higher organisms, redox regulation in lower organisms is geared towards promoting oxidative stress resistance. Given that autophagy is not important for oxidative stress resistance in *S. pombe*, there could be no evolutionary pressure for this organism to develop a mechanism to increase autophagic activity during oxidative stress.

While redox regulation of DUBs and ULPs has not been thoroughly investigated, it has great potential as a regulatory mechanism. Firstly, DUBs are emerging as important regulators of many cellular processes, including the cell cycle (Song and Rape, 2008; Stegmeier et al., 2007). Secondly, there are vast numbers of DUBs and ULPs in eukaryotes, and each enzyme may act on specific substrates. Therefore, redox-regulation of a specific DUB or ULP could affect only a small number of proteins, making this an ideal mechanism for regulating activity or stability of specific proteins. For example, specific SENPs form inhibitory intermolecular disulfides during oxidative stress in both yeast and mammals, which could increase the SUMOylation of particular substrates (Xu et al., 2008). This is potentially a far more precise mechanism than redox regulation of Uba2 and Ubc9, which affects global SUMOylation (Bossis and Melchior, 2006). Furthermore, oxidative inactivation of an Urm1-specific ULP may explain why oxidative stress increases urmylation in *S. cerevisiae* and mammals (Goehring et al., 2003b; Van der Veen et al., 2011). Thus, in addition to investigating redox regulation of E1s and E2s, it is important that regulation of DUBs and ULPs is also investigated, in order to provide a full impression of how ubiquitin/Ubl conjugation is regulated under different conditions.

Post-translational modification may also represent a mechanism through which ubiquitin/Ubl conjugation can be regulated during stress. Although redox regulation of Atg4 could not be detected in *S. pombe*, Atg4 phosphorylation appeared to be decreased following nitrogen starvation. This is the first documentation of post-translational modification of Atg4, and it is tempting to speculate that this plays a role in the induction of autophagy. How post-translational modification of Atg4 affects the function of the enzyme remains unknown. However, interestingly, the deacetylation of many Atg proteins is important for the induction of autophagy in mammalian cells (Lee et al., 2008; Lee and Finkel, 2009; Salminen and Kaarniranta, 2009), and it is possible that dephosphorylation of Atg4 is similarly important for autophagy induction in *S. pombe*. Furthermore, the possible regulation of Atg4 activity by post-translational modification may have implications for the regulation of other ubiquitin/Ubl conjugation pathways. Indeed, it is conceivable that other enzymes controlling ubiquitin/Ubl conjugation could be post-translationally modified to regulate their activity in response to various stimuli, including oxidative stress, and this may represent another means of controlling substrate modification.

In addition, there are other possible mechanisms through which ubiquitin/Ubl conjugation can be promoted in response to specific stimuli. Firstly, post-translational modification of substrates has important roles in the regulation of ubiquitin/Ubl conjugation. For example, in *S. cerevisiae*, Sic1 is phosphorylated by Cdks during G1 phase, thus regulating the timing of ubiquitination and degradation of the protein (Feldman et al., 1997). It is possible that changes in substrate modification can also regulate ubiquitin/Ubl conjugation during stress. Interestingly, Rum1 is phosphorylated by Sty1 *in vitro* (Matsuoka et al., 2002), and hence may represent an example of a mechanism by which ROS can indirectly regulate protein activity and stability, via enzymes that are activated in response to stress. Secondly, the control of gene expression can influence ubiquitin/Ubl conjugation to substrates. For example, H<sub>2</sub>O<sub>2</sub> has been shown to increase the expression of genes encoding specific E2s and E3s during oxidative stress (Li et al., 2003b). This would be expected to increase ubiquitination of specific proteins. Indeed, the expression of E3 regulatory subunits is regulated in response to many different stimuli, and oscillates during the cell cycle to control progression via the degradation of cyclins, Cdks and Cdkis (Nakayama and Nakayama, 2006). Clearly, many regulatory mechanisms have to be considered and integrated together in order to give a full impression of how ubiquitin/Ubl conjugation is affected by particular stimuli.

### **6.3. Summary and Future Perspectives.**

In summary, the investigations in this thesis have demonstrated the importance of ubiquitin/Ubl conjugation in responses to oxidative stress in *S. pombe*, as well as in other eukaryotes, and have shown that redox regulation of specific ubiquitin/Ubl conjugation events may be a conserved or species-specific response to elevated concentrations of ROS. Furthermore, theories have been proposed as to why certain enzymes in these pathways are redox-regulated, while others are not.

In order to gain further insight into how ubiquitin/Ubl conjugation pathways are regulated during oxidative stress, the redox-sensitivities of E1s, E2s, E3s, DUBs and ULPs all required further investigation. Some of these enzymes may be highly sensitive to oxidation, while others could be less sensitive, resulting in distinct effects on ubiquitin/Ubl conjugation at different levels of oxidative stress. The techniques used in this thesis could easily be reproduced for this purpose. Alternatively, other approaches could be used to identify enzymes that are oxidised *in vivo*. In recent years, molecular probes have been designed to bind oxidised proteins *in vivo*, allowing these proteins to be purified and identified (Poole and Nelson, 2008). Once redox-sensitive enzymes have been identified, the consequences of their inhibition could be investigated. However, a major challenge for the field is to identify the substrates that are modified by specific enzymes. For example, redox regulation of Ubc15 could not be related to the effects of oxidative stress on the ubiquitination of downstream substrates, as the substrates of Ubc15 are unknown. Furthermore, the targets of DUBs and ULPs are largely unknown. Progress depends on identifying these substrates and monitoring their modification by ubiquitin/Ubls. Subsequently, the effects of oxidative stress on ubiquitin/Ubl conjugation to specific substrates could be investigated.

In conclusion, redox regulation of ubiquitin/Ubl conjugation influences many different processes in eukaryotic cells, and is important for responses to oxidative stress in higher and lower eukaryotes. Further investigations are required to shed more light on how ubiquitin/Ubl conjugation protects cells from oxidative damage, and the extent to which this is conserved from yeast to human.

### Extra References

- Feldman, R.M., Correll, C.C., Kaplan, K.B., and Deshaies, R.J. (1997). A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. *Cell* 91, 221-230.
- Jahngen-Hodge, J., Obin, M.S., Gong, X., Shang, F., Nowell, T.R., Jr., Gong, J., Abasi, H., Blumberg, J., and Taylor, A. (1997). Regulation of ubiquitin-conjugating enzymes by glutathione following oxidative stress. *The Journal of biological chemistry* 272, 28218-28226.
- Matsuoka, K., Kiyokawa, N., Taguchi, T., Matsui, J., Suzuki, T., Mimori, K., Nakajima, H., Takenouchi, H., Weiran, T., Katagiri, Y.U., *et al.* (2002). Rum1, an inhibitor of cyclin-dependent kinase in fission yeast, is negatively regulated by mitogen-activated protein kinase-mediated phosphorylation at Ser and Thr residues. *European journal of biochemistry / FEBS* 269, 3511-3521.
- Quinn, J., Findlay, V.J., Dawson, K., Millar, J.B., Jones, N., Morgan, B.A., and Toone, W.M. (2002). Distinct regulatory proteins control the graded transcriptional response to increasing H<sub>2</sub>O<sub>2</sub> levels in fission yeast *Schizosaccharomyces pombe*. *Molecular biology of the cell* 13, 805-816.

## References

- Adams, D.O., Johnson, W.J., and Marino, P.A. (1982). Mechanisms of target recognition and destruction in macrophage-mediated tumor cytotoxicity. *Federation Proceedings* 41, 2212-2221.
- al-Khodairy, F., Enoch, T., Hagan, I.M., and Carr, A.M. (1995). The *Schizosaccharomyces pombe* *hus5* gene encodes a ubiquitin conjugating enzyme required for normal mitosis. *J Cell Sci* 108, 475-486.
- Alfa, C.E., Gallagher, I.M., and Hyams, J.S. (1993). Antigen localization in fission yeast. *Methods Cell Biol* 37, 201-222.
- Alao, J.P., and Sunnerhagen, P. (2008). Rad3 and Sty1 function in *Schizosaccharomyces pombe*: an integrated response to DNA damage and environmental stress? *Mol Microbiology* 68, 246-254.
- Ankar, J., and Sistonen, L. (2007). SUMO: getting it on. *Biochemical Society Transactions* 35, 1409-1413.
- Arico, S., Petiot, A., Bauvy, C., Dubbelhuis, P.F., Meijer, A.J., Codogno, P., and Ogier-Denis, E. (2001). The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. *J Biol Chem* 276, 35243-35246.
- Ashraf, S.S., Sochacka, E., Cain, R., Guenther, R., Malkiewicz, A., and Agris, P.F. (1999). Single atom modification (O-->S) of tRNA confers ribosome binding. *RNA* 5, 188-194.
- Asp, E., Nilsson, D., and Sunnerhagen, P. (2008). Fission yeast mitogen-activated protein kinase Sty1 interacts with translation factors. *Eukaryotic Cell* 7, 328-338.
- Azad, M.B., Chen, Y., and Gibson, S.B. (2009). Regulation of autophagy by reactive oxygen species (ROS): implications for cancer progression and treatment. *Antioxidants & Redox Signaling* 11, 777-790.
- Bae, Y.S., Kang, S.W., Seo, M.S., Baines, I.C., Tekle, E., Chock, P.B., and Rhee, S.G. (1997). Epidermal growth factor (EGF)-induced generation of hydrogen peroxide. Role in EGF receptor-mediated tyrosine phosphorylation. *J Biol Chem* 272, 217-221.
- Bae, Y.S., Sung, J.Y., Kim, O.S., Kim, Y.J., Hur, K.C., Kazlauskas, A., and Rhee, S.G. (2000). Platelet-derived growth factor-induced H<sub>2</sub>O<sub>2</sub> production requires the activation of phosphatidylinositol 3-kinase. *J Biol Chem* 275, 10527-10531.
- Balaban, R.S., Nemoto, S., and Finkel, T. (2005). Mitochondria, oxidants, and aging. *Cell* 120, 483-495.

- Barba, G., Soto, T., Madrid, M., Nunez, A., Vicente, J., Gacto, M., and Cansado, J. (2008). Activation of the cell integrity pathway is channelled through diverse signalling elements in fission yeast. *Cellular Signalling* 20, 748-757.
- Barford, D. (2004). The role of cysteine residues as redox-sensitive regulatory switches. *Curr Opin Struct Biol* 14, 679-686.
- Barrett, W.C., DeGnore, J.P., Konig, S., Fales, H.M., Keng, Y.F., Zhang, Z.Y., Yim, M.B., and Chock, P.B. (1999). Regulation of PTP1B via glutathionylation of the active site cysteine 215. *Biochemistry* 38, 6699-6705.
- Bayer, P., Arndt, A., Metzger, S., Mahajan, R., Melchior, F., Jaenicke, R., and Becker, J. (1998). Structure determination of the small ubiquitin-related modifier SUMO-1. *J Mol Biol* 280, 275-286.
- Beckman, K.B., and Ames, B.N. (1997). Oxidative decay of DNA. *J Biol Chem* 272, 19633-19636.
- Bedford, L., Lowe, J., Dick, L.R., Mayer, R.J., and Brownell, J.E. (2011). Ubiquitin-like protein conjugation and the ubiquitin-proteasome system as drug targets. *Nat Rev Drug Disc* 10, 29-46.
- Benito, J., Martin-Castellanos, C., and Moreno, S. (1998). Regulation of the G1 phase of the cell cycle by periodic stabilization and degradation of the p25<sup>rum1</sup> CDK inhibitor. *EMBO* 17, 482-497.
- Bensaad, K., Cheung, E.C., and Vousden, K.H. (2009). Modulation of intracellular ROS levels by TIGAR controls autophagy. *EMBO* 28, 3015-3026.
- Berlanga, J.J., Rivero, D., Martin, R., Herrero, S., Moreno, S., and de Haro, C. (2010). Role of mitogen-activated protein kinase Sty1 in regulation of eukaryotic initiation factor 2 $\alpha$  kinases in response to environmental stress in *Schizosaccharomyces pombe*. *Eukaryotic Cell* 9, 194-207.
- Berlett, B.S., and Stadtman, E.R. (1997). Protein oxidation in aging, disease, and oxidative stress. *J Biol Chem* 272, 20313-20316.
- Birnboim, H.C., and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nuc Acid Res* 7, 1513-1523.
- Biteau, B., Labarre, J., and Toledano, M.B. (2003). ATP-dependent reduction of cysteine-sulphinic acid by *S. cerevisiae* sulphiredoxin. *Nature* 425, 980-984.
- Bjork, G.R., Huang, B., Persson, O.P., and Bystrom, A.S. (2007). A conserved modified wobble nucleoside (mcm5s2U) in lysyl-tRNA is required for viability in yeast. *RNA* 13, 1245-1255.

- Bjorkoy, G., Lamark, T., Brech, A., Outzen, H., Perander, M., Overvatn, A., Stenmark, H., and Johansen, T. (2005). p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol* 171, 603-614.
- Bossis, G., Malnou, C.E., Farras, R., Andermarcher, E., Hipskind, R., Rodriguez, M., Schmidt, D., Muller, S., Jariel-Encontre, I., and Piechaczyk, M. (2005). Down-regulation of c-Fos/c-Jun AP-1 dimer activity by sumoylation. *Mol Cell Biol* 25, 6964-6979.
- Bossis, G., and Melchior, F. (2006). Regulation of SUMOylation by reversible oxidation of SUMO conjugating enzymes. *Mol Cell* 21, 349-357.
- Bosu, D.R., and Kipreos, E.T. (2008). Cullin-RING ubiquitin ligases: global regulation and activation cycles. *Cell Division* 3, 7.
- Boveris, A., and Chance, B. (1973). The mitochondrial generation of hydrogen peroxide. General properties and effect of hyperbaric oxygen. *Biochem J* 134, 707-716.
- Bozonet, S.M., Findlay, V.J., Day, A.M., Cameron, J., Veal, E.A., and Morgan, B.A. (2005). Oxidation of a eukaryotic 2-Cys peroxiredoxin is a molecular switch controlling the transcriptional response to increasing levels of hydrogen peroxide. *J Biol Chem* 280, 23319-23327.
- Brewster, J.L., de Valoir, T., Dwyer, N.D., Winter, E., and Gustin, M.C. (1993). An osmosensing signal transduction pathway in yeast. *Science* 259, 1760-1763.
- Buck, V., Quinn, J., Soto Pino, T., Martin, H., Saldanha, J., Makino, K., Morgan, B.A., and Millar, J.B. (2001). Peroxide sensors for the fission yeast stress-activated mitogen-activated protein kinase pathway. *Mol Biol Cell* 12, 407-419.
- Budovskaya, Y.V., Stephan, J.S., Reggiori, F., Klionsky, D.J., and Herman, P.K. (2004). The Ras/cAMP-dependent protein kinase signaling pathway regulates an early step of the autophagy process in *Saccharomyces cerevisiae*. *J Biol Chem* 279, 20663-20671.
- Burhans, W.C., and Heintz, N.H. (2009). The cell cycle is a redox cycle: linking phase-specific targets to cell fate. *Free Rad Biol & Med* 47, 1282-1293.
- Butterfield, D.A., and Boyd-Kimball, D. (2004). Amyloid beta-peptide(1-42) contributes to the oxidative stress and neurodegeneration found in Alzheimer disease brain. *Brain Pathol* 14, 426-432.
- Buzek, J., Latonen, L., Kurki, S., Peltonen, K., and Laiho, M. (2002). Redox state of tumor suppressor p53 regulates its sequence-specific DNA binding in DNA-damaged cells by cysteine 277. *Nuc Scid Res* 30, 2340-2348.



- Byers, B., and Goetsch, L. (1974). Duplication of spindle plaques and integration of the yeast cell cycle. *Cold Spring Harbor symposia on quantitative biology* 38, 123-131.
- Capili, A.D., and Lima, C.D. (2007). Taking it step by step: mechanistic insights from structural studies of ubiquitin/ubiquitin-like protein modification pathways. *Curr Opin Struct Biol* 17, 726-735.
- Cash, T.P., Pan, Y., and Simon, M.C. (2007). Reactive oxygen species and cellular oxygen sensing. *Free Rad Biol & Med* 43, 1219-1225.
- Castillo, E.A., Ayte, J., Chiva, C., Moldon, A., Carrascal, M., Abian, J., Jones, N., and Hidalgo, E. (2002). Diethylmaleate activates the transcription factor Pap1 by covalent modification of critical cysteine residues. *Mol Microbiol* 45, 243-254.
- Cerutti, P.A. (1985). Prooxidant states and tumor promotion. *Science* 227, 375-381.
- Chae, H.Z., Kim, H.J., Kang, S.W., and Rhee, S.G. (1999). Characterization of three isoforms of mammalian peroxiredoxin that reduce peroxides in the presence of thioredoxin. *Diabetes Res* 45, 101-112.
- Chakravarthi, S., Jessop, C.E., and Bulleid, N.J. (2006). The role of glutathione in disulphide bond formation and endoplasmic-reticulum-generated oxidative stress. *EMBO Reports* 7, 271-275.
- Chang, T.S., Jeong, W., Choi, S.Y., Yu, S., Kang, S.W., and Rhee, S.G. (2002). Regulation of peroxiredoxin I activity by Cdc2-mediated phosphorylation. *J Biol Chem* 277, 25370-25376.
- Chen, D., Wilkinson, C.R., Watt, S., Penkett, C.J., Toone, W.M., Jones, N., and Bahler, J. (2008a). Multiple pathways differentially regulate global oxidative stress responses in fission yeast. *Mol Biol Cell* 19, 308-317.
- Chen, X.Q., Du, X., Liu, J., Balasubramanian, M.K., and Balasundaram, D. (2004). Identification of genes encoding putative nucleoporins and transport factors in the fission yeast *Schizosaccharomyces pombe*: a deletion analysis. *Yeast* 21, 495-509.
- Chen, Y. (2007). The enzymes in ubiquitin-like post-translational modifications. *Bioscience Trends* 1, 16-25.
- Chen, Y., McMillan-Ward, E., Kong, J., Israels, S.J., and Gibson, S.B. (2007). Mitochondrial electron-transport-chain inhibitors of complexes I and II induce autophagic cell death mediated by reactive oxygen species. *J Cell Sci* 120, 4155-4166.
- Chen, Y., McMillan-Ward, E., Kong, J., Israels, S.J., and Gibson, S.B. (2008b). Oxidative stress induces autophagic cell death independent of apoptosis in transformed and cancer cells. *Cell Death Diff* 15, 171-182.

- Chen, Z.J., and Sun, L.J. (2009). Nonproteolytic functions of ubiquitin in cell signaling. *Mol Cell* 33, 275-286.
- Chevtzoff, C., Yoboue, E.D., Galinier, A., Casteilla, L., Daignan-Fornier, B., Rigoulet, M., and Devin, A. (2010). Reactive oxygen species-mediated regulation of mitochondrial biogenesis in the yeast *Saccharomyces cerevisiae*. *J Biol Chem* 285, 1733-1742.
- Chiu, Y.H., Sun, Q., and Chen, Z.J. (2007). E1-L2 activates both ubiquitin and FAT10. *Mol Cell* 27, 1014-1023.
- Choi, H., Kim, S., Mukhopadhyay, P., Cho, S., Woo, J., Storz, G., and Ryu, S.E. (2001). Structural basis of the redox switch in the OxyR transcription factor. *Cell* 105, 103-113.
- Choi, M.H., Lee, I.K., Kim, G.W., Kim, B.U., Han, Y.H., Yu, D.Y., Park, H.S., Kim, K.Y., Lee, J.S., Choi, C., *et al.* (2005). Regulation of PDGF signalling and vascular remodelling by peroxiredoxin II. *Nature* 435, 347-353.
- Cosgrove, J.P., Borish, E.T., Church, D.F., and Pryor, W.A. (1985). The metal-mediated formation of hydroxyl radical by aqueous extracts of cigarette tar. *Biochem Biophys Res Comm* 132, 390-396.
- Craven, R.A., Griffiths, D.J., Sheldrick, K.S., Randall, R.E., Hagan, I.M., and Carr, A.M. (1998). Vectors for the expression of tagged proteins in *Schizosaccharomyces pombe*. *Gene* 221, 59-68.
- Cuervo, A.M. (2004). Autophagy: in sickness and in health. *Trends in cell biology* 14, 70-77.
- D'Autreaux, B., and Toledano, M.B. (2007). ROS as signalling molecules: mechanisms that generate specificity in ROS homeostasis. *Nat Rev Mol Cell Biol* 8, 813-824.
- Dasso, M. (2008). Emerging roles of the SUMO pathway in mitosis. *Cell division* 3, 5.
- David, Y., Ziv, T., Admon, A., and Navon, A. (2010). The E2 ubiquitin-conjugating enzymes direct polyubiquitination to preferred lysines. *J Biol Chem* 285, 8595-8604.
- Day, A.M., Brown, J.D., Taylor, S.R., Rand, J.D., Morgan, B.A., and Veal, E.A. (2012). Inactivation of a peroxiredoxin by hydrogen peroxide is critical for thioredoxin-mediated repair of oxidized proteins and cell survival. *Mol Cell* 45, 398-408.
- Day, A.M., and Veal, E.A. (2010). Hydrogen peroxide-sensitive cysteines in the Sty1 MAPK regulate the transcriptional response to oxidative stress. *J Biol Chem* 285, 7505-7516.
- Degols, G., Shiozaki, K., and Russell, P. (1996). Activation and regulation of the Spc1 stress-activated protein kinase in *Schizosaccharomyces pombe*. *Mol Cell Biol* 16, 2870-2877.

- Delaunay, A., Isnard, A.D., and Toledano, M.B. (2000). H<sub>2</sub>O<sub>2</sub> sensing through oxidation of the Yap1 transcription factor. *EMBO J* 19, 5157-5166.
- Delaunay, A., Pflieger, D., Barrault, M.B., Vinh, J., and Toledano, M.B. (2002). A thiol peroxidase is an H<sub>2</sub>O<sub>2</sub> receptor and redox-transducer in gene activation. *Cell* 111, 471-481.
- Denu, J.M., and Dixon, J.E. (1998). Protein tyrosine phosphatases: mechanisms of catalysis and regulation. *Curr Opin Chem Biol* 2, 633-641.
- Desterro, J.M., Rodriguez, M.S., and Hay, R.T. (1998). SUMO-1 modification of I $\kappa$ B $\alpha$  inhibits NF- $\kappa$ B activation. *Mol Cell* 2, 233-239.
- Desterro, J.M., Thomson, J., and Hay, R.T. (1997). Ubc9 conjugates SUMO but not ubiquitin. *FEBS letters* 417, 297-300.
- Dewez, M., Bauer, F., Dieu, M., Raes, M., Vandenhoute, J., and Hermand, D. (2008). The conserved Wobble uridine tRNA thiolase Ctu1-Ctu2 is required to maintain genome integrity. *PNAS* 105, 5459-5464.
- DeYulia, G.J., Jr., Carcamo, J.M., Borquez-Ojeda, O., Shelton, C.C., and Golde, D.W. (2005). Hydrogen peroxide generated extracellularly by receptor-ligand interaction facilitates cell signaling. *PNAS* 102, 5044-5049.
- Dieckhoff, P., Bolte, M., Sancak, Y., Braus, G.H., and Irniger, S. (2004). Smt3/SUMO and Ubc9 are required for efficient APC/C-mediated proteolysis in budding yeast. *Mol Microbiol* 51, 1375-1387.
- Djavaheri-Mergny, M., Amelotti, M., Mathieu, J., Besancon, F., Bauvy, C., Souquere, S., Pierron, G., and Codogno, P. (2006). NF- $\kappa$ B activation represses tumor necrosis factor- $\alpha$ -induced autophagy. *J Biol Chem* 281, 30373-30382.
- Duda, D.M., Walden, H., Sfondouris, J., and Schulman, B.A. (2005). Structural analysis of Escherichia coli ThiF. *J Mol Biol* 349, 774-786.
- Dunand-Sauthier, I., Walker, C.A., Narasimhan, J., Pearce, A.K., Wek, R.C., and Humphrey, T.C. (2005). Stress-activated protein kinase pathway functions to support protein synthesis and translational adaptation in response to environmental stress in fission yeast. *Eukaryotic Cell* 4, 1785-1793.
- Dye, B.T., and Schulman, B.A. (2007). Structural mechanisms underlying posttranslational modification by ubiquitin-like proteins. *Ann Rev Biophys Biomol Struct* 36, 131-150.
- Eletr, Z.M., Huang, D.T., Duda, D.M., Schulman, B.A., and Kuhlman, B. (2005). E2 conjugating enzymes must disengage from their E1 enzymes before E3-dependent ubiquitin and ubiquitin-like transfer. *Nat Struct Mol Biol* 12, 933-934.

Feldman, R.M., Correll, C.C., Kaplan, K.B., and Deshaies, R.J. (1997). A complex of Cdc4p, Skp1p, and Cdc53p/cullin catalyzes ubiquitination of the phosphorylated CDK inhibitor Sic1p. *Cell* 91, 221-230.

Fernandes, L., Rodrigues-Pousada, C., and Struhl, K. (1997). Yap, a novel family of eight bZIP proteins in *Saccharomyces cerevisiae* with distinct biological functions. *Mol Cell Biol* 17, 6982-6993.

Filomeni, G., Rotilio, G., and Ciriolo, M.R. (2005). Disulfide relays and phosphorylative cascades: partners in redox-mediated signaling pathways. *Cell Death Diff* 12, 1555-1563.

Finkel, T., and Holbrook, N.J. (2000). Oxidants, oxidative stress and the biology of ageing. *Nature* 408, 239-247.

Fomenko, D.E., Koc, A., Agisheva, N., Jacobsen, M., Kaya, A., Malinouski, M., Rutherford, J.C., Siu, K.L., Jin, D.Y., Winge, D.R., *et al.* (2011). Thiol peroxidases mediate specific genome-wide regulation of gene expression in response to hydrogen peroxide. *PNAS* 108, 2729-2734.

Foreman, J., Demidchik, V., Bothwell, J.H., Mylona, P., Miedema, H., Torres, M.A., Linstead, P., Costa, S., Brownlee, C., Jones, J.D., *et al.* (2003). Reactive oxygen species produced by NADPH oxidase regulate plant cell growth. *Nature* 422, 442-446.

Fujino, G., Noguchi, T., Takeda, K., and Ichijo, H. (2006). Thioredoxin and protein kinases in redox signaling. *Sem Cancer Biol* 16, 427-435.

Furukawa, K., Mizushima, N., Noda, T., and Ohsumi, Y. (2000). A protein conjugation system in yeast with homology to biosynthetic enzyme reaction of prokaryotes. *J Biol Chem* 275, 7462-7465.

Gallogly, M.M., and Mieryl, J.J. (2007). Mechanisms of reversible protein glutathionylation in redox signaling and oxidative stress. *Curr Opin Pharm* 7, 381-391.

Gareau, J.R., and Lima, C.D. (2010). The SUMO pathway: emerging mechanisms that shape specificity, conjugation and recognition. *Nat Rev Mol Cell Biol* 11, 861-871.

Geeraert, C., Ratier, A., Pfisterer, S.G., Perdiz, D., Cantaloube, I., Rouault, A., Patingre, S., Proikas-Cezanne, T., Codogno, P., and Pous, C. (2010). Starvation-induced hyperacetylation of tubulin is required for the stimulation of autophagy by nutrient deprivation. *J Biol Chem* 285, 24184-24194.

Geisler, S., Holmstrom, K.M., Skujat, D., Fiesel, F.C., Rothfuss, O.C., Kahle, P.J., and Springer, W. (2010). PINK1/Parkin-mediated mitophagy is dependent on VDAC1 and p62/SQSTM1. *Nat Cell Biol* 12, 119-131.

- Geiss-Friedlander, R., and Melchior, F. (2007). Concepts in sumoylation: a decade on. *Nat Rev Mol Cell Biol* 8, 947-956.
- Geng, J., and Klionsky, D.J. (2008). The Atg8 and Atg12 ubiquitin-like conjugation systems in macroautophagy. 'Protein modifications: beyond the usual suspects' review series. *EMBO reports* 9, 859-864.
- Ghezzi, P. (2005). Regulation of protein function by glutathionylation. *Free Rad Res* 39, 573-580.
- Girdwood, D., Xirodimas, D.P., and Gordon, C. (2011). The essential functions of NEDD8 are mediated via distinct surface regions, and not by polyneddylation in *Schizosaccharomyces pombe*. *PloS one* 6, e20089.
- Gius, D., Botero, A., Shah, S., and Curry, H.A. (1999). Intracellular oxidation/reduction status in the regulation of transcription factors NF-kappaB and AP-1. *Toxicology letters* 106, 93-106.
- Glickman, M.H., and Ciechanover, A. (2002). The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. *Physiological reviews* 82, 373-428.
- Glotzer, M., Murray, A.W., and Kirschner, M.W. (1991). Cyclin is degraded by the ubiquitin pathway. *Nature* 349, 132-138.
- Goebel, M.G., Yochem, J., Jentsch, S., McGrath, J.P., Varshavsky, A., and Byers, B. (1988). The yeast cell cycle gene CDC34 encodes a ubiquitin-conjugating enzyme. *Science* 241, 1331-1335.
- Goehring, A.S., Rivers, D.M., and Sprague, G.F., Jr. (2003a). Attachment of the ubiquitin-related protein Urm1p to the antioxidant protein Ahp1p. *Eukaryotic Cell* 2, 930-936.
- Goehring, A.S., Rivers, D.M., and Sprague, G.F., Jr. (2003b). Urm1ylation: a ubiquitin-like pathway that functions during invasive growth and budding in yeast. *Mol Biol Cell* 14, 4329-4341.
- Gong, L., and Yeh, E.T. (1999). Identification of the activating and conjugating enzymes of the NEDD8 conjugation pathway. *J Biol Chem* 274, 12036-12042.
- Groettrup, M., Pelzer, C., Schmidtke, G., and Hofmann, K. (2008). Activating the ubiquitin family: UBA6 challenges the field. *Trends Biochem Sci* 33, 230-237.
- Guardavaccaro, D., and Pagano, M. (2004). Oncogenic aberrations of cullin-dependent ubiquitin ligases. *Oncogene* 23, 2037-2049.
- Gupta, S., Campbell, D., Derijard, B., and Davis, R.J. (1995). Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science* 267, 389-393.

- Gutierrez, G.J., and Ronai, Z. (2006). Ubiquitin and SUMO systems in the regulation of mitotic checkpoints. *Trends Biochem Sci* 31, 324-332.
- Gutscher, M., Sobotta, M.C., Wabnitz, G.H., Ballikaya, S., Meyer, A.J., Samstag, Y., and Dick, T.P. (2009). Proximity-based protein thiol oxidation by H<sub>2</sub>O<sub>2</sub>-scavenging peroxidases. *J Biol Chem* 284, 31532-31540.
- Haglund, K., Sigismund, S., Polo, S., Szymkiewicz, I., Di Fiore, P.P., and Dikic, I. (2003). Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. *Nat Cell Biol* 5, 461-466.
- Hainaut, P., and Milner, J. (1993). Redox modulation of p53 conformation and sequence-specific DNA binding in vitro. *Cancer Res* 53, 4469-4473.
- Halliwell, B., and Gutteridge, J.M. (1990). Role of free radicals and catalytic metal ions in human disease: an overview. *Methods in Enzymology* 186, 1-85.
- Hanada, T., Noda, N.N., Satomi, Y., Ichimura, Y., Fujioka, Y., Takao, T., Inagaki, F., and Ohsumi, Y. (2007). The Atg12-Atg5 conjugate has a novel E3-like activity for protein lipidation in autophagy. *J Biol Chem* 282, 37298-37302.
- Haracska, L., Torres-Ramos, C.A., Johnson, R.E., Prakash, S., and Prakash, L. (2004). Opposing effects of ubiquitin conjugation and SUMO modification of PCNA on replicational bypass of DNA lesions in *Saccharomyces cerevisiae*. *Mol Cell Biol* 24, 4267-4274.
- Hardeland, U., Steinacher, R., Jiricny, J., and Schar, P. (2002). Modification of the human thymine-DNA glycosylase by ubiquitin-like proteins facilitates enzymatic turnover. *EMBO* 21, 1456-1464.
- Harman, D. (1956). Aging: a theory based on free radical and radiation chemistry. *J Gerontology* 11, 298-300.
- Harreman, M., Taschner, M., Sigurdsson, S., Anindya, R., Reid, J., Somesh, B., Kong, S.E., Banks, C.A., Conaway, R.C., Conaway, J.W., *et al.* (2009). Distinct ubiquitin ligases act sequentially for RNA polymerase II polyubiquitylation. *PNAS* 106, 20705-20710.
- Hay, R.T. (2005). SUMO: a history of modification. *Mol Cell* 18, 1-12.
- Heideker, J., Perry, J.J., and Boddy, M.N. (2009). Genome stability roles of SUMO-targeted ubiquitin ligases. *DNA Repair* 8, 517-524.
- Herrmann, J., Lerman, L.O., and Lerman, A. (2007). Ubiquitin and ubiquitin-like proteins in protein regulation. *Circ Res* 100, 1276-1291.
- Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. *Ann Rev Biochem* 67, 425-479.

Hidalgo, E., and Demple, B. (1994). An iron-sulfur center essential for transcriptional activation by the redox-sensing SoxR protein. *EMBO J* 13, 138-146.

Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., and Pease, L.R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 77, 51-59.

Hochstrasser, M. (2000). Evolution and function of ubiquitin-like protein-conjugation systems. *Nat Cell Biol* 2, E153-157.

Hochstrasser, M. (2009). Origin and function of ubiquitin-like proteins. *Nature* 458, 422-429.

Hoege, C., Pfander, B., Moldovan, G.L., Pyrowolakis, G., and Jentsch, S. (2002). RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature* 419, 135-141.

Huang, B., Lu, J., and Bystrom, A.S. (2008). A genome-wide screen identifies genes required for formation of the wobble nucleoside 5-methoxycarbonylmethyl-2-thiouridine in *Saccharomyces cerevisiae*. *RNA* 14, 2183-2194.

Huang, D.T., Miller, D.W., Mathew, R., Cassell, R., Holton, J.M., Roussel, M.F., and Schulman, B.A. (2004). A unique E1-E2 interaction required for optimal conjugation of the ubiquitin-like protein NEDD8. *Nat Struct Mol Biol* 11, 927-935.

Huang, D.T., Paydar, A., Zhuang, M., Waddell, M.B., Holton, J.M., and Schulman, B.A. (2005). Structural basis for recruitment of Ubc12 by an E2 binding domain in NEDD8's E1. *Mol Cell* 17, 341-350.

Huang, J., Canadien, V., Lam, G.Y., Steinberg, B.E., Dinaiuer, M.C., Magalhaes, M.A., Glogauer, M., Grinstein, S., and Brumell, J.H. (2009). Activation of antibacterial autophagy by NADPH oxidases. *PNAS* 106, 6226-6231.

Huang, L., Kinnucan, E., Wang, G., Beaudenon, S., Howley, P.M., Huibregtse, J.M., and Pavletich, N.P. (1999). Structure of an E6AP-UbcH7 complex: insights into ubiquitination by the E2-E3 enzyme cascade. *Science* 286, 1321-1326.

Huang, T.T., Nijman, S.M., Mirchandani, K.D., Galardy, P.J., Cohn, M.A., Haas, W., Gygi, S.P., Ploegh, H.L., Bernards, R., and D'Andrea, A.D. (2006). Regulation of monoubiquitinated PCNA by DUB autocleavage. *Nat Cell Biol* 8, 339-347.

Humbard, M.A., Miranda, H.V., Lim, J.M., Krause, D.J., Pritz, J.R., Zhou, G., Chen, S., Wells, L., and Maupin-Furlow, J.A. (2010). Ubiquitin-like small archaeal modifier proteins (SAMPs) in *Haloferax volcanii*. *Nature* 463, 54-60.

Humphries, K.M., Juliano, C., and Taylor, S.S. (2002). Regulation of cAMP-dependent protein kinase activity by glutathionylation. *J Biol Chem* 277, 43505-43511.

Hutchins, M.U., Veenhuis, M., and Klionsky, D.J. (1999). Peroxisome degradation in *Saccharomyces cerevisiae* is dependent on machinery of macroautophagy and the Cvt pathway. *J Cell Sci* 112 ( Pt 22), 4079-4087.

Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., Takagi, M., Matsumoto, K., Miyazono, K., and Gotoh, Y. (1997). Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science* 275, 90-94.

Ichimura, Y., Kirisako, T., Takao, T., Satomi, Y., Shimonishi, Y., Ishihara, N., Mizushima, N., Tanida, I., Kominami, E., Ohsumi, M., *et al.* (2000). A ubiquitin-like system mediates protein lipidation. *Nature* 408, 488-492.

Ikeda, F., and Dikic, I. (2008). Atypical ubiquitin chains: new molecular signals. 'Protein Modifications: Beyond the Usual Suspects' review series. *EMBO reports* 9, 536-542.

Ikner, A., and Shiozaki, K. (2005). Yeast signaling pathways in the oxidative stress response. *Mutation Res* 569, 13-27.

Imoto, K., Kukidome, D., Nishikawa, T., Matsuhisa, T., Sonoda, K., Fujisawa, K., Yano, M., Motoshima, H., Taguchi, T., Tsuruzoe, K., *et al.* (2006). Impact of mitochondrial reactive oxygen species and apoptosis signal-regulating kinase 1 on insulin signaling. *Diabetes* 55, 1197-1204.

Iwai, K., Naganuma, A., and Kuge, S. (2010). Peroxiredoxin Ahp1 acts as a receptor for alkylhydroperoxides to induce disulfide bond formation in the Cad1 transcription factor. *J Biol Chem* 285, 10597-10604.

Iyer, L.M., Burroughs, A.M., and Aravind, L. (2006). The prokaryotic antecedents of the ubiquitin-signaling system and the early evolution of ubiquitin-like beta-grasp domains. *Genome Biology* 7, R60.

Jahngen-Hodge, J., Obin, M.S., Gong, X., Shang, F., Nowell, T.R., Jr., Gong, J., Abasi, H., Blumberg, J., and Taylor, A. (1997). Regulation of ubiquitin-conjugating enzymes by glutathione following oxidative stress. *J Biol Chem* 272, 28218-28226.

Jang, H.H., Lee, K.O., Chi, Y.H., Jung, B.G., Park, S.K., Park, J.H., Lee, J.R., Lee, S.S., Moon, J.C., Yun, J.W., *et al.* (2004). Two enzymes in one; two yeast peroxiredoxins display oxidative stress-dependent switching from a peroxidase to a molecular chaperone function. *Cell* 117, 625-635.

Johnson, E.S., and Blobel, G. (1997). Ubc9p is the conjugating enzyme for the ubiquitin-like protein Smt3p. *J Biol Chem* 272, 26799-26802.



Johnson, E.S., Schwienhorst, I., Dohmen, R.J., and Blobel, G. (1997). The ubiquitin-like protein Smt3p is activated for conjugation to other proteins by an Aos1p/Uba2p heterodimer. *EMBO J* 16, 5509-5519.

Kadowaki, H., Nishitoh, H., Urano, F., Sadamitsu, C., Matsuzawa, A., Takeda, K., Masutani, H., Yodoi, J., Urano, Y., Nagano, T., *et al.* (2005). Amyloid beta induces neuronal cell death through ROS-mediated ASK1 activation. *Cell Death Diff* 12, 19-24.

Kamada, Y., Funakoshi, T., Shintani, T., Nagano, K., Ohsumi, M., and Ohsumi, Y. (2000). Tor-mediated induction of autophagy via an Apg1 protein kinase complex. *J Cell Biol* 150, 1507-1513.

Kamata, H., Honda, S., Maeda, S., Chang, L., Hirata, H., and Karin, M. (2005). Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell* 120, 649-661.

Kanki, T., and Klionsky, D.J. (2008). Mitophagy in yeast occurs through a selective mechanism. *J Biol Chem* 283, 32386-32393.

Kato, T., Jr., Okazaki, K., Murakami, H., Stettler, S., Fantes, P.A., and Okayama, H. (1996). Stress signal, mediated by a Hog1-like MAP kinase, controls sexual development in fission yeast. *FEBS letters* 378, 207-212.

Kawakami, T., Chiba, T., Suzuki, T., Iwai, K., Yamanaka, K., Minato, N., Suzuki, H., Shimbara, N., Hidaka, Y., Osaka, F., *et al.* (2001). NEDD8 recruits E2-ubiquitin to SCF E3 ligase. *EMBO J* 20, 4003-4012.

Kennedy, N.J., and Davis, R.J. (2003). Role of JNK in tumor development. *Cell Cycle* 2, 199-201.

Kerscher, O., Felberbaum, R., and Hochstrasser, M. (2006). Modification of proteins by ubiquitin and ubiquitin-like proteins. *Ann Rev Cell Dev* 22, 159-180.

Kiel, J.A. (2010). Autophagy in unicellular eukaryotes. *Philosophical Transactions of the Royal Society of London Series B, Biological sciences* 365, 819-830.

Kim, S.O., Merchant, K., Nudelman, R., Beyer, W.F., Jr., Keng, T., DeAngelo, J., Hausladen, A., and Stamler, J.S. (2002). OxyR: a molecular code for redox-related signaling. *Cell* 109, 383-396.

King, R.W., Deshaies, R.J., Peters, J.M., and Kirschner, M.W. (1996). How proteolysis drives the cell cycle. *Science* 274, 1652-1659.

Kirisako, T., Baba, M., Ishihara, N., Miyazawa, K., Ohsumi, M., Yoshimori, T., Noda, T., and Ohsumi, Y. (1999). Formation process of autophagosome is traced with Apg8/Aut7p in yeast. *J Cell Biol* 147, 435-446.

Kirisako, T., Ichimura, Y., Okada, H., Kabeya, Y., Mizushima, N., Yoshimori, T., Ohsumi, M., Takao, T., Noda, T., and Ohsumi, Y. (2000). The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. *J Cell Biol* 151, 263-276.

Kissova, I., Deffieu, M., Manon, S., and Camougrand, N. (2004). Uth1p is involved in the autophagic degradation of mitochondria. *J Biol Chem* 279, 39068-39074.

Kitamura, K., Taki, M., Tanaka, N., and Yamashita, I. (2011). Fission yeast Ubr1 ubiquitin ligase influences the oxidative stress response via degradation of active Pap1 bZIP transcription factor in the nucleus. *Mol Microbiology* 80, 739-755.

Kleiger, G., Saha, A., Lewis, S., Kuhlman, B., and Deshaies, R.J. (2009). Rapid E2-E3 assembly and disassembly enable processive ubiquitylation of cullin-RING ubiquitin ligase substrates. *Cell* 139, 957-968.

Klionsky, D.J. (2005). The molecular machinery of autophagy: unanswered questions. *J Cell Sci* 118, 7-18.

Klionsky, D.J., Cregg, J.M., Dunn, W.A., Jr., Emr, S.D., Sakai, Y., Sandoval, I.V., Sibirny, A., Subramani, S., Thumm, M., Veenhuis, M., *et al.* (2003). A unified nomenclature for yeast autophagy-related genes. *Dev Cell* 5, 539-545.

Kohda, T.A., Tanaka, K., Konomi, M., Sato, M., Osumi, M., and Yamamoto, M. (2007). Fission yeast autophagy induced by nitrogen starvation generates a nitrogen source that drives adaptation processes. *Genes To Cells* 12, 155-170.

Komatsu, M., Tanida, I., Ueno, T., Ohsumi, M., Ohsumi, Y., and Kominami, E. (2001). The C-terminal region of an Apg7p/Cvt2p is required for homodimerization and is essential for its E1 activity and E1-E2 complex formation. *J Biol Chem* 276, 9846-9854.

Kornitzer, D., and Ciechanover, A. (2000). Modes of regulation of ubiquitin-mediated protein degradation. *J Cell Phys* 182, 1-11.

Krepinsky, K., and Leimkuhler, S. (2007). Site-directed mutagenesis of the active site loop of the rhodanese-like domain of the human molybdopterin synthase sulfurase MOCS3. Major differences in substrate specificity between eukaryotic and bacterial homologs. *FEBS* 274, 2778-2787.

Kruse, J.P., and Gu, W. (2009). MSL2 promotes Mdm2-independent cytoplasmic localization of p53. *J Biol Chem* 284, 3250-3263.

Kudo, N., Taoka, H., Toda, T., Yoshida, M., and Horinouchi, S. (1999). A novel nuclear export signal sensitive to oxidative stress in the fission yeast transcription factor Pap1. *J Biol Chem* 274, 15151-15158.

- Kuge, S., Jones, N., and Nomoto, A. (1997). Regulation of yAP-1 nuclear localization in response to oxidative stress. *EMBO J* 16, 1710-1720.
- Kumar, A., Wu, H., Collier-Hyams, L.S., Hansen, J.M., Li, T., Yamoah, K., Pan, Z.Q., Jones, D.P., and Neish, A.S. (2007). Commensal bacteria modulate cullin-dependent signaling via generation of reactive oxygen species. *EMBO J* 26, 4457-4466.
- Kumar, A., Wu, H., Collier-Hyams, L.S., Kwon, Y.M., Hanson, J.M., and Neish, A.S. (2009). The bacterial fermentation product butyrate influences epithelial signaling via reactive oxygen species-mediated changes in cullin-1 neddylation. *J Immunol* 182, 538-546.
- Kunzler, M., and Hurt, E.C. (1998). Cse1p functions as the nuclear export receptor for importin alpha in yeast. *FEBS letters* 433, 185-190.
- Kurz, T., Chou, Y.C., Willems, A.R., Meyer-Schaller, N., Hecht, M.L., Tyers, M., Peter, M., and Sicheri, F. (2008). Dcn1 functions as a scaffold-type E3 ligase for cullin neddylation. *Mol Cell* 29, 23-35.
- Kutay, U., Bischoff, F.R., Kostka, S., Kraft, R., and Gorlich, D. (1997). Export of importin alpha from the nucleus is mediated by a specific nuclear transport factor. *Cell* 90, 1061-1071.
- Labib, K., and Moreno, S. (1996). rum1: a CDK inhibitor regulating G1 progression in fission yeast. *Trends Cell Biol* 6, 62-66.
- Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-685.
- Lake, M.W., Wuebbens, M.M., Rajagopalan, K.V., and Schindelin, H. (2001). Mechanism of ubiquitin activation revealed by the structure of a bacterial MoeB-MoaD complex. *Nature* 414, 325-329.
- Lambeth, J.D. (2004). NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol* 4, 181-189.
- Landles, C., and Bates, G.P. (2004). Huntingtin and the molecular pathogenesis of Huntington's disease. Fourth in molecular medicine review series. *EMBO reports* 5, 958-963.
- Lawrence, C.L., Jones, N., and Wilkinson, C.R. (2009). Stress-induced phosphorylation of *S. pombe* Atf1 abrogates its interaction with F box protein Fbh1. *Curr Biol* 19, 1907-1911.
- Leck, Y.C., Choo, Y.Y., Tan, C.Y., Smith, P.G., and Hagen, T. (2010). Biochemical and cellular effects of inhibiting Nedd8 conjugation. *Biochem Biophys Res Comm* 398, 588-593.

- Lee, G.W., Melchior, F., Matunis, M.J., Mahajan, R., Tian, Q., and Anderson, P. (1998a). Modification of Ran GTPase-activating protein by the small ubiquitin-related modifier SUMO-1 requires Ubc9, an E2-type ubiquitin-conjugating enzyme homologue. *J Biol Chem* 273, 6503-6507.
- Lee, I., and Schindelin, H. (2008). Structural insights into E1-catalyzed ubiquitin activation and transfer to conjugating enzymes. *Cell* 134, 268-278.
- Lee, I.H., Cao, L., Mostoslavsky, R., Lombard, D.B., Liu, J., Bruns, N.E., Tsokos, M., Alt, F.W., and Finkel, T. (2008). A role for the NAD-dependent deacetylase Sirt1 in the regulation of autophagy. *PNAS* 105, 3374-3379.
- Lee, I.H., and Finkel, T. (2009). Regulation of autophagy by the p300 acetyltransferase. *J Biol Chem* 284, 6322-6328.
- Lee, S.R., Kwon, K.S., Kim, S.R., and Rhee, S.G. (1998b). Reversible inactivation of protein-tyrosine phosphatase 1B in A431 cells stimulated with epidermal growth factor. *J Biol Chem* 273, 15366-15372.
- Lee, S.R., Yang, K.S., Kwon, J., Lee, C., Jeong, W., and Rhee, S.G. (2002). Reversible inactivation of the tumor suppressor PTEN by H<sub>2</sub>O<sub>2</sub>. *J Biol Chem* 277, 20336-20342.
- Lehmann, C., Begley, T.P., and Ealick, S.E. (2006). Structure of the Escherichia coli ThiS-ThiF complex, a key component of the sulfur transfer system in thiamin biosynthesis. *Biochemistry* 45, 11-19.
- Leidecker, O., Matic, I., Mahata, B., Pion, E., and Xirodimas, D.P. (2012). The ubiquitin E1 enzyme Ube1 mediates NEDD8 activation under diverse stress conditions. *Cell Cycle* 11, 1142-1150.
- Leidel, S., Pedrioli, P.G., Bucher, T., Brost, R., Costanzo, M., Schmidt, A., Aebersold, R., Boone, C., Hofmann, K., and Peter, M. (2009). Ubiquitin-related modifier Urm1 acts as a sulphur carrier in thiolation of eukaryotic transfer RNA. *Nature* 458, 228-232.
- Levine, B., and Kroemer, G. (2008). Autophagy in the pathogenesis of disease. *Cell* 132, 27-42.
- Levonen, A.L., Patel, R.P., Brookes, P., Go, Y.M., Jo, H., Parthasarathy, S., Anderson, P.G., and Darley-Usmar, V.M. (2001). Mechanisms of cell signaling by nitric oxide and peroxynitrite: from mitochondria to MAP kinases. *Antioxidants & Redox Signaling* 3, 215-229.
- Li, J., Stouffs, M., Serrander, L., Banfi, B., Bettiol, E., Charnay, Y., Steger, K., Krause, K.H., and Jacon, M.E. (2006). The NADPH oxidase NOX4 drives cardiac differentiation: Role in regulating cardiac transcription factors and MAP kinase activation. *Mol Biol Cell* 17, 3978-3988.

- Li, M., Brooks, C.L., Wu-Baer, F., Chen, D., Baer, R., and Gu, W. (2003a). Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. *Science* 302, 1972-1975.
- Li, Y.P., Chen, Y., Li, A.S., and Reid, M.B. (2003b). Hydrogen peroxide stimulates ubiquitin-conjugating activity and expression of genes for specific E2 and E3 proteins in skeletal muscle myotubes. *Am J Cell Phys* 285, C806-812.
- Liakopoulos, D., Doenges, G., Matuschewski, K., and Jentsch, S. (1998). A novel protein modification pathway related to the ubiquitin system. *EMBO* 17, 2208-2214.
- Liochev, S.I., and Fridovich, I. (1992). Fumarase C, the stable fumarase of *Escherichia coli*, is controlled by the soxRS regulon. *PNAS* 89, 5892-5896.
- Liu, B., Chen, Y., and St Clair, D.K. (2008). ROS and p53: a versatile partnership. *Free Rad Biol Med* 44, 1529-1535.
- Liu, Y., and Min, W. (2002). Thioredoxin promotes ASK1 ubiquitination and degradation to inhibit ASK1-mediated apoptosis in a redox activity-independent manner. *Circ Res* 90, 1259-1266.
- Livingstone, C., Patel, G., and Jones, N. (1995). ATF-2 contains a phosphorylation-dependent transcriptional activation domain. *EMBO* 14, 1785-1797.
- Loewith, R., Hubberstey, A., and Young, D. (2000). Skh1, the MEK component of the mkh1 signaling pathway in *Schizosaccharomyces pombe*. *J Cell Sci* 113 ( Pt 1), 153-160.
- Lois, L.M., and Lima, C.D. (2005). Structures of the SUMO E1 provide mechanistic insights into SUMO activation and E2 recruitment to E1. *EMBO* 24, 439-451.
- Lopez-Aviles, S., Grande, M., Gonzalez, M., Helgesen, A.L., Alemany, V., Sanchez-Piris, M., Bachs, O., Millar, J.B., and Aligue, R. (2005). Inactivation of the Cdc25 phosphatase by the stress-activated Srk1 kinase in fission yeast. *Mol Cell* 17, 49-59.
- Lopez-Aviles, S., Lambea, E., Moldon, A., Grande, M., Fajardo, A., Rodriguez-Gabriel, M.A., Hidalgo, E., and Aligue, R. (2008). Activation of Srk1 by the mitogen-activated protein kinase Sty1/Spc1 precedes its dissociation from the kinase and signals its degradation. *Mol Biol Cell* 19, 1670-1679.
- Lyapina, S., Cope, G., Shevchenko, A., Serino, G., Tsuge, T., Zhou, C., Wolf, D.A., Wei, N., and Deshaies, R.J. (2001). Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome. *Science* 292, 1382-1385.
- Madrid, M., Nunez, A., Soto, T., Vicente-Soler, J., Gacto, M., and Cansado, J. (2007). Stress-activated protein kinase-mediated down-regulation of the cell integrity pathway

mitogen-activated protein kinase Pmk1p by protein phosphatases. *Mol Biol Cell* 18, 4405-4419.

Madrid, M., Soto, T., Franco, A., Paredes, V., Vicente, J., Hidalgo, E., Gacto, M., and Cansado, J. (2004). A cooperative role for Atf1 and Pap1 in the detoxification of the oxidative stress induced by glucose deprivation in *Schizosaccharomyces pombe*. *J Biol Chem* 279, 41594-41602.

Madrid, M., Soto, T., Khong, H.K., Franco, A., Vicente, J., Perez, P., Gacto, M., and Cansado, J. (2006). Stress-induced response, localization, and regulation of the Pmk1 cell integrity pathway in *Schizosaccharomyces pombe*. *J Biol Chem* 281, 2033-2043.

Mahajan, R., Delphin, C., Guan, T., Gerace, L., and Melchior, F. (1997). A small ubiquitin-related polypeptide involved in targeting RanGAP1 to nuclear pore complex protein RanBP2. *Cell* 88, 97-107.

Marshall, C.J. (1994). MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr Opin Gene Dev* 4, 82-89.

Matsuoka, K., Kiyokawa, N., Taguchi, T., Matsui, J., Suzuki, T., Mimori, K., Nakajima, H., Takenouchi, H., Weiran, T., Katagiri, Y.U., *et al.* (2002). Rum1, an inhibitor of cyclin-dependent kinase in fission yeast, is negatively regulated by mitogen-activated protein kinase-mediated phosphorylation at Ser and Thr residues. *Eur J Biochem / FEBS* 269, 3511-3521.

Matsuzawa, A., and Ichijo, H. (2008). Redox control of cell fate by MAP kinase: physiological roles of ASK1-MAP kinase pathway in stress signaling. *Biochimica et Biophysica Acta* 1780, 1325-1336.

Matunis, M.J., Coutavas, E., and Blobel, G. (1996). A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. *J Cell Biol* 135, 1457-1470.

Mendenhall, M.D. (1993). An inhibitor of p34CDC28 protein kinase activity from *Saccharomyces cerevisiae*. *Science* 259, 216-219.

Michael, W.M., and Newport, J. (1998). Coupling of mitosis to the completion of S phase through Cdc34-mediated degradation of Wee1. *Science* 282, 1886-1889.

Mikawa, T., Kanoh, J., and Ishikawa, F. (2010). Fission yeast Vps1 and Atg8 contribute to oxidative stress resistance. *Genes To Cells : devoted to molecular & cellular mechanisms*.

Millar, J.B., Buck, V., and Wilkinson, M.G. (1995). Pyp1 and Pyp2 PTPases dephosphorylate an osmosensing MAP kinase controlling cell size at division in fission yeast. *Gene Dev* 9, 2117-2130.

- Miranda, H.V., Nembhard, N., Su, D., Hepowit, N., Krause, D.J., Pritz, J.R., Phillips, C., Soll, D., and Maupin-Furlow, J.A. (2011). E1- and ubiquitin-like proteins provide a direct link between protein conjugation and sulfur transfer in archaea. *PNAS* *108*, 4417-4422.
- Miyamoto, Y., Saiwaki, T., Yamashita, J., Yasuda, Y., Kotera, I., Shibata, S., Shigeta, M., Hiraoka, Y., Haraguchi, T., and Yoneda, Y. (2004). Cellular stresses induce the nuclear accumulation of importin alpha and cause a conventional nuclear import block. *J Cell Biol* *165*, 617-623.
- Mizushima, N., Levine, B., Cuervo, A.M., and Klionsky, D.J. (2008). Autophagy fights disease through cellular self-digestion. *Nature* *451*, 1069-1075.
- Mizushima, N., Noda, T., and Ohsumi, Y. (1999). Apg16p is required for the function of the Apg12p-Apg5p conjugate in the yeast autophagy pathway. *EMBO* *18*, 3888-3896.
- Mizushima, N., Noda, T., Yoshimori, T., Tanaka, Y., Ishii, T., George, M.D., Klionsky, D.J., Ohsumi, M., and Ohsumi, Y. (1998). A protein conjugation system essential for autophagy. *Nature* *395*, 395-398.
- Mizushima, N., Yoshimori, T., and Ohsumi, Y. (2002). Mouse Apg10 as an Apg12-conjugating enzyme: analysis by the conjugation-mediated yeast two-hybrid method. *FEBS letters* *532*, 450-454.
- Mohideen, F., Capili, A.D., Bilimoria, P.M., Yamada, T., Bonni, A., and Lima, C.D. (2009). A molecular basis for phosphorylation-dependent SUMO conjugation by the E2 UBC9. *Nat Struct Mol Biol* *16*, 945-952.
- Moreno, S., Klar, A., and Nurse, P. (1991). Molecular genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods In Enzymology* *194*, 795-823.
- Mukaiyama, H., Kajiwar, S., Hosomi, A., Giga-Hama, Y., Tanaka, N., Nakamura, T., and Takegawa, K. (2009). Autophagy-deficient *Schizosaccharomyces pombe* mutants undergo partial sporulation during nitrogen starvation. *Microbiology* *155*, 3816-3826.
- Mukaiyama, H., Nakase, M., Nakamura, T., Kakinuma, Y., and Takegawa, K. (2010). Autophagy in the fission yeast *Schizosaccharomyces pombe*. *FEBS letters* *584*, 1327-1334.
- Mukherjee, S., and Orth, K. (2008). Microbiology. A protein pupylation paradigm. *Science* *322*, 1062-1063.
- Nadeau, P.J., Charette, S.J., Toledano, M.B., and Landry, J. (2007). Disulfide Bond-mediated multimerization of Ask1 and its reduction by thioredoxin-1 regulate

H(2)O(2)-induced c-Jun NH(2)-terminal kinase activation and apoptosis. *Mol Biol Cell* 18, 3903-3913.

Nakai, Y., Nakai, M., and Hayashi, H. (2008). Thio-modification of yeast cytosolic tRNA requires a ubiquitin-related system that resembles bacterial sulfur transfer systems. *J Biol Chem* 283, 27469-27476.

Nakatogawa, H., Suzuki, K., Kamada, Y., and Ohsumi, Y. (2009). Dynamics and diversity in autophagy mechanisms: lessons from yeast. *Nat Rev Mol Cell Biol* 10, 458-467.

Nakayama, K.I., and Nakayama, K. (2006). Ubiquitin ligases: cell-cycle control and cancer. *Nat Rev Cancer* 6, 369-381.

Narendra, D., Tanaka, A., Suen, D.F., and Youle, R.J. (2008). Parkin is recruited selectively to impaired mitochondria and promotes their autophagy. *J Cell Biol* 183, 795-803.

Nefsky, B., and Beach, D. (1996). Pub1 acts as an E6-AP-like protein ubiquitin ligase in the degradation of cdc25. *EMBO* 15, 1301-1312.

Nemoto, T., Tanida, I., Tanida-Miyake, E., Minematsu-Ikeguchi, N., Yokota, M., Ohsumi, M., Ueno, T., and Kominami, E. (2003). The mouse APG10 homologue, an E2-like enzyme for Apg12p conjugation, facilitates MAP-LC3 modification. *J Biol Chem* 278, 39517-39526.

Nguyen, A.N., and Shiozaki, K. (1999). Heat-shock-induced activation of stress MAP kinase is regulated by threonine- and tyrosine-specific phosphatases. *Gene Dev* 13, 1653-1663.

Nielsen, I.S., Nielsen, O., Murray, J.M., and Thon, G. (2002). The fission yeast ubiquitin-conjugating enzymes UbcP3, Ubc15, and Rhp6 affect transcriptional silencing of the mating-type region. *Eukaryotic Cell* 1, 613-625.

Nishizawa, M., Kawasumi, M., Fujino, M., and Toh-e, A. (1998). Phosphorylation of sic1, a cyclin-dependent kinase (Cdk) inhibitor, by Cdk including Pho85 kinase is required for its prompt degradation. *Mol Biol Cell* 9, 2393-2405.

Noda, N.N., Ohsumi, Y., and Inagaki, F. (2009). ATG systems from the protein structural point of view. *Chem Rev* 109, 1587-1598.

Noda, T., and Ohsumi, Y. (1998). Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. *J Biol Chem* 273, 3963-3966.

Noguchi, T., Takeda, K., Matsuzawa, A., Saegusa, K., Nakano, H., Gohda, J., Inoue, J., and Ichijo, H. (2005). Recruitment of tumor necrosis factor receptor-associated factor



family proteins to apoptosis signal-regulating kinase 1 signalosome is essential for oxidative stress-induced cell death. *J Biol Chem* 280, 37033-37040.

Noma, A., Sakaguchi, Y., and Suzuki, T. (2009). Mechanistic characterization of the sulfur-relay system for eukaryotic 2-thiouridine biogenesis at tRNA wobble positions. *Nuc Acid Res* 37, 1335-1352.

Oberley, T.D. (2002). Oxidative damage and cancer. *Am J Pathology* 160, 403-408.

Obin, M., Shang, F., Gong, X., Handelman, G., Blumberg, J., and Taylor, A. (1998). Redox regulation of ubiquitin-conjugating enzymes: mechanistic insights using the thiol-specific oxidant diamide. *FASEB J* 12, 561-569.

Okamoto, K., Kondo-Okamoto, N., and Ohsumi, Y. (2009). A landmark protein essential for mitophagy: Atg32 recruits the autophagic machinery to mitochondria. *Autophagy* 5, 1203-1205.

Okazaki, S., Naganuma, A., and Kuge, S. (2005). Peroxiredoxin-mediated redox regulation of the nuclear localization of Yap1, a transcription factor in budding yeast. *Antioxidants & Redox Signaling* 7, 327-334.

Onodera, J., and Ohsumi, Y. (2004). Ald6p is a preferred target for autophagy in yeast, *Saccharomyces cerevisiae*. *J Biol Chem* 279, 16071-16076.

Onodera, J., and Ohsumi, Y. (2005). Autophagy is required for maintenance of amino acid levels and protein synthesis under nitrogen starvation. *J Biol Chem* 280, 31582-31586.

Osaka, F., Kawasaki, H., Aida, N., Saeki, M., Chiba, T., Kawashima, S., Tanaka, K., and Kato, S. (1998). A new NEDD8-ligating system for cullin-4A. *Gene Dev* 12, 2263-2268.

Osaka, F., Saeki, M., Katayama, S., Aida, N., Toh, E.A., Kominami, K., Toda, T., Suzuki, T., Chiba, T., Tanaka, K., *et al.* (2000). Covalent modifier NEDD8 is essential for SCF ubiquitin-ligase in fission yeast. *EMBO* 19, 3475-3484.

Pagano, M. (1997). Cell cycle regulation by the ubiquitin pathway. *FASEB J* 11, 1067-1075.

Pan, Z.Q., Kentsis, A., Dias, D.C., Yamoah, K., and Wu, K. (2004). Nedd8 on cullin: building an expressway to protein destruction. *Oncogene* 23, 1985-1997.

Pankiv, S., Clausen, T.H., Lamark, T., Brech, A., Bruun, J.A., Outzen, H., Overvatn, A., Bjorkoy, G., and Johansen, T. (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J Biol Chem* 282, 24131-24145.

- Park, H.S., Lee, S.H., Park, D., Lee, J.S., Ryu, S.H., Lee, W.J., Rhee, S.G., and Bae, Y.S. (2004). Sequential activation of phosphatidylinositol 3-kinase, beta Pix, Rac1, and Nox1 in growth factor-induced production of H<sub>2</sub>O<sub>2</sub>. *Mol Cell Biol* 24, 4384-4394.
- Pascual, M.B., Mata-Cabana, A., Florencio, F.J., Lindahl, M., and Cejudo, F.J. (2010). Overoxidation of 2-Cys peroxiredoxin in prokaryotes: cyanobacterial 2-Cys peroxiredoxins sensitive to oxidative stress. *J Biol Chem* 285, 34485-34492.
- Pattingre, S., Espert, L., Biard-Piechaczyk, M., and Codogno, P. (2008). Regulation of macroautophagy by mTOR and Beclin 1 complexes. *Biochimie* 90, 313-323.
- Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X.H., Mizushima, N., Packer, M., Schneider, M.D., and Levine, B. (2005). Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* 122, 927-939.
- Pearce, M.J., Mintseris, J., Ferreyra, J., Gygi, S.P., and Darwin, K.H. (2008). Ubiquitin-like protein involved in the proteasome pathway of *Mycobacterium tuberculosis*. *Science* 322, 1104-1107.
- Pedrioli, P.G., Leidel, S., and Hofmann, K. (2008). Urm1 at the crossroad of modifications. *EMBO reports* 9, 1196-1202.
- Petersen, J., and Hagan, I.M. (2005). Polo kinase links the stress pathway to cell cycle control and tip growth in fission yeast. *Nature* 435, 507-512.
- Petersen, J., and Nurse, P. (2007). TOR signalling regulates mitotic commitment through the stress MAP kinase pathway and the Polo and Cdc2 kinases. *Nat Cell Biol* 9, 1263-1272.
- Petroski, M.D., and Deshaies, R.J. (2005). Function and regulation of cullin-RING ubiquitin ligases. *Nat Rev Mol Cell Biol* 6, 9-20.
- Petroski, M.D., and Deshaies, R.J. (2005). Mechanism of lysine 48-linked ubiquitin-chain synthesis by the cullin-RING ubiquitin-ligase complex SCF-Cdc34. *Cell* 123, 1107-1120.
- Pichler, A., Knipscheer, P., Oberhofer, E., van Dijk, W.J., Korner, R., Olsen, J.V., Jentsch, S., Melchior, F., and Sixma, T.K. (2005). SUMO modification of the ubiquitin-conjugating enzyme E2-25K. *Nat Struct Mol Biol* 12, 264-269.
- Plafker, K.S., Nguyen, L., Barneche, M., Mirza, S., Crawford, D., and Plafker, S.M. (2010). The ubiquitin-conjugating enzyme UbcM2 can regulate the stability and activity of the antioxidant transcription factor Nrf2. *J Biol Chem* 285, 23064-23074.
- Pollycove, M., and Feinendegen, L.E. (2003). Radiation-induced versus endogenous DNA damage: possible effect of inducible protective responses in mitigating

endogenous damage. *Human & Experimental Toxicology* 22, 290-306; Discussion 307, 315-297, 319-223.

Quinn, J., Findlay, V.J., Dawson, K., Millar, J.B., Jones, N., Morgan, B.A., and Toone, W.M. (2002). Distinct regulatory proteins control the graded transcriptional response to increasing H<sub>2</sub>O<sub>2</sub> levels in fission yeast *Schizosaccharomyces pombe*. *Mol Biol Cell* 13, 805-816.

Rabut, G., and Peter, M. (2008). Function and regulation of protein neddylation. 'Protein modifications: beyond the usual suspects' review series. *EMBO reports* 9, 969-976.

Radoshevich, L., Murrow, L., Chen, N., Fernandez, E., Roy, S., Fung, C., and Debnath, J. (2010). ATG12 conjugation to ATG3 regulates mitochondrial homeostasis and cell death. *Cell* 142, 590-600.

Rainwater, R., Parks, D., Anderson, M.E., Tegtmeyer, P., and Mann, K. (1995). Role of cysteine residues in regulation of p53 function. *Mol Cell Biol* 15, 3892-3903.

Reggiori, F., and Klionsky, D.J. (2002). Autophagy in the eukaryotic cell. *Eukaryotic Cell* 1, 11-21.

Reverter, D., and Lima, C.D. (2005). Insights into E3 ligase activity revealed by a SUMO-RanGAP1-Ubc9-Nup358 complex. *Nature* 435, 687-692.

Rhee, S.G. (2006). Cell signaling. H<sub>2</sub>O<sub>2</sub>, a necessary evil for cell signaling. *Science* 312, 1882-1883.

Rhee, S.G., Bae, Y.S., Lee, S.R., and Kwon, J. (2000). Hydrogen peroxide: a key messenger that modulates protein phosphorylation through cysteine oxidation. *Science's STKE : signal transduction knowledge environment* 2000, pe1.

Rhee, S.G., Chang, T.S., Bae, Y.S., Lee, S.R., and Kang, S.W. (2003). Cellular regulation by hydrogen peroxide. *JASN* 14, S211-215.

Rhee, S.G., Jeong, W., Chang, T.S., and Woo, H.A. (2007). Sulfiredoxin, the cysteine sulfinic acid reductase specific to 2-Cys peroxiredoxin: its discovery, mechanism of action, and biological significance. *Kidney International Supplement*, S3-8.

Rhee, S.G., Kang, S.W., Jeong, W., Chang, T.S., Yang, K.S., and Woo, H.A. (2005). Intracellular messenger function of hydrogen peroxide and its regulation by peroxiredoxins. *Curr Opin Cell Biol* 17, 183-189.

Rhee, S.G., Woo, H.A., Kil, I.S., and Bae, S.H. (2012). Peroxiredoxin functions as a peroxidase and a regulator and sensor of local peroxides. *J Biol Chem* 287, 4403-4410.

Ribet, D., Hamon, M., Gouin, E., Nahori, M.A., Impens, F., Neyret-Kahn, H., Gevaert, K., Vandekerckhove, J., Dejean, A., and Cossart, P. (2010). *Listeria monocytogenes* impairs SUMOylation for efficient infection. *Nature* 464, 1192-1195.

Rodrigo-Brenni, M.C., Foster, S.A., and Morgan, D.O. (2010). Catalysis of lysine 48-specific ubiquitin chain assembly by residues in E2 and ubiquitin. *Mol Cell* 39, 548-559.

Rodriguez-Gabriel, M.A., Burns, G., McDonald, W.H., Martin, V., Yates, J.R., 3rd, Bahler, J., and Russell, P. (2003). RNA-binding protein Csx1 mediates global control of gene expression in response to oxidative stress. *EMBO* 22, 6256-6266.

Rodriguez-Gabriel, M.A., and Russell, P. (2005). Distinct signaling pathways respond to arsenite and reactive oxygen species in *Schizosaccharomyces pombe*. *Eukaryotic Cell* 4, 1396-1402.

Rodriguez-Gabriel, M.A., Watt, S., Bahler, J., and Russell, P. (2006). Upf1, an RNA helicase required for nonsense-mediated mRNA decay, modulates the transcriptional response to oxidative stress in fission yeast. *Mol Cell Biol* 26, 6347-6356.

Rodriguez-Gabriel, M.A., Watt, S., Bahler, J., and Russell, P. (2006). Upf1, an RNA helicase required for nonsense-mediated mRNA decay, modulates the transcriptional response to oxidative stress in fission yeast. *Mol Cell Biol* 26, 6347-6356.

Rodriguez-Hernandez, A., Cordero, M.D., Salviati, L., Artuch, R., Pineda, M., Briones, P., Gomez Izquierdo, L., Cotan, D., Navas, P., and Sanchez-Alcazar, J.A. (2009). Coenzyme Q deficiency triggers mitochondria degradation by mitophagy. *Autophagy* 5, 19-32.

Rodriguez, M.S., Dargemont, C., and Hay, R.T. (2001). SUMO-1 conjugation in vivo requires both a consensus modification motif and nuclear targeting. *J Biol Chem* 276, 12654-12659.

Rubio-Teixeira, M. (2007). Urmylelation controls Nil1p and Gln3p-dependent expression of nitrogen-catabolite repressed genes in *Saccharomyces cerevisiae*. *FEBS letters* 581, 541-550.

Rudolph, M.J., Wuebbens, M.M., Rajagopalan, K.V., and Schindelin, H. (2001). Crystal structure of molybdopterin synthase and its evolutionary relationship to ubiquitin activation. *Nat Struct Biol* 8, 42-46.

Sablina, A.A., Budanov, A.V., Ilyinskaya, G.V., Agapova, L.S., Kravchenko, J.E., and Chumakov, P.M. (2005). The antioxidant function of the p53 tumor suppressor. *Nat Med* 11, 1306-1313.

Saha, A., and Deshaies, R.J. (2008). Multimodal activation of the ubiquitin ligase SCF by Nedd8 conjugation. *Mol Cell* 32, 21-31.

Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998). Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J* 17, 2596-2606.

Sajiki, K., Hatanaka, M., Nakamura, T., Takeda, K., Shimanuki, M., Yoshida, T., Hanyu, Y., Hayashi, T., Nakaseko, Y., and Yanagida, M. (2009). Genetic control of cellular quiescence in *S. pombe*. *J Cell Sci* 122, 1418-1429.

Salinas, A.E., and Wong, M.G. (1999). Glutathione S-transferases--a review. *Curr Med Chem* 6, 279-309.

Salminen, A., and Kaarniranta, K. (2009). SIRT1: regulation of longevity via autophagy. *Cellular Signalling* 21, 1356-1360.

Samejima, I., Mackie, S., and Fantes, P.A. (1997). Multiple modes of activation of the stress-responsive MAP kinase pathway in fission yeast. *EMBO J* 16, 6162-6170.

Sanchez-Diaz, A., Gonzalez, I., Arellano, M., and Moreno, S. (1998). The Cdk inhibitors p25rum1 and p40SIC1 are functional homologues that play similar roles in the regulation of the cell cycle in fission and budding yeast. *J Cell Sci* 111 ( Pt 6), 843-851.

Sanchez-Piris, M., Posas, F., Alemany, V., Winge, I., Hidalgo, E., Bachs, O., and Aligue, R. (2002). The serine/threonine kinase Cmk2 is required for oxidative stress response in fission yeast. *J Biol Chem* 277, 17722-17727.

Sanso, M., Gogol, M., Ayte, J., Seidel, C., and Hidalgo, E. (2008). Transcription factors Pcr1 and Atf1 have distinct roles in stress- and Sty1-dependent gene regulation. *Eukaryotic Cell* 7, 826-835.

Savitsky, P.A., and Finkel, T. (2002). Redox regulation of Cdc25C. *J Biol Chem* 277, 20535-20540.

Scherz-Shouval, R., and Elazar, Z. (2007). ROS, mitochondria and the regulation of autophagy. *Trends Cell Biol* 17, 422-427.

Scherz-Shouval, R., Shvets, E., Fass, E., Shorer, H., Gil, L., and Elazar, Z. (2007). Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. *EMBO J* 26, 1749-1760.

Schmitz, J., Chowdhury, M.M., Hanzelmann, P., Nimtz, M., Lee, E.Y., Schindelin, H., and Leimkuhler, S. (2008). The sulfurtransferase activity of Uba4 presents a link between ubiquitin-like protein conjugation and activation of sulfur carrier proteins. *Biochemistry* 47, 6479-6489.

Schuller, C., Brewster, J.L., Alexander, M.R., Gustin, M.C., and Ruis, H. (1994). The HOG pathway controls osmotic regulation of transcription via the stress response element (STRE) of the *Saccharomyces cerevisiae* *CTT1* gene. *EMBO J* 13, 4382-4389.

Schulman, B.A., and Harper, J.W. (2009). Ubiquitin-like protein activation by E1 enzymes: the apex for downstream signalling pathways. *Nat Rev Mol Cell Biol* 10, 319-331.

Schwob, E., Bohm, T., Mendenhall, M.D., and Nasmyth, K. (1994). The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in *S. cerevisiae*. *Cell* 79, 233-244.

Sengar, A.S., Markley, N.A., Marini, N.J., and Young, D. (1997). Mkh1, a MEK kinase required for cell wall integrity and proper response to osmotic and temperature stress in *Schizosaccharomyces pombe*. *Mol Cell Biol* 17, 3508-3519.

Shayeghi, M., Doe, C.L., Tavassoli, M., and Watts, F.Z. (1997). Characterisation of *Schizosaccharomyces pombe rad31*, a UBA-related gene required for DNA damage tolerance. *Nuc Acid Res* 25, 1162-1169.

Shieh, J.C., Wilkinson, M.G., Buck, V., Morgan, B.A., Makino, K., and Millar, J.B. (1997). The Mcs4 response regulator coordinately controls the stress-activated Wak1-Wis1-Sty1 MAP kinase pathway and fission yeast cell cycle. *Gene Dev* 11, 1008-1022.

Shieh, J.C., Wilkinson, M.G., and Millar, J.B. (1998). The Win1 mitotic regulator is a component of the fission yeast stress-activated Sty1 MAPK pathway. *Mol Biol Cell* 9, 311-322.

Shin, J.A., Choi, E.S., Kim, H.S., Ho, J.C., Watts, F.Z., Park, S.D., and Jang, Y.K. (2005). SUMO modification is involved in the maintenance of heterochromatin stability in fission yeast. *Mol Cell* 19, 817-828.

Shintani, T., and Klionsky, D.J. (2004). Autophagy in health and disease: a double-edged sword. *Science* 306, 990-995.

Shintani, T., Mizushima, N., Ogawa, Y., Matsuura, A., Noda, T., and Ohsumi, Y. (1999). Apg10p, a novel protein-conjugating enzyme essential for autophagy in yeast. *EMBO J* 18, 5234-5241.

Shiozaki, K., and Russell, P. (1995). Cell-cycle control linked to extracellular environment by MAP kinase pathway in fission yeast. *Nature* 378, 739-743.

Shiozaki, K., and Russell, P. (1996). Conjugation, meiosis, and the osmotic stress response are regulated by Spc1 kinase through Atf1 transcription factor in fission yeast. *Gene Dev* 10, 2276-2288.

- Shiozaki, K., Shiozaki, M., and Russell, P. (1997). Mcs4 mitotic catastrophe suppressor regulates the fission yeast cell cycle through the Wlk1-Wis1-Spc1 kinase cascade. *Mol Biol Cell* 8, 409-419.
- Shiozaki, K., Shiozaki, M., and Russell, P. (1998). Heat stress activates fission yeast Spc1/StyI MAPK by a MEKK-independent mechanism. *Mol Biol Cell* 9, 1339-1349.
- Skowyra, D., Craig, K.L., Tyers, M., Elledge, S.J., and Harper, J.W. (1997). F-box proteins are receptors that recruit phosphorylated substrates to the SCF ubiquitin-ligase complex. *Cell* 91, 209-219.
- Smith, D.A., Toone, W.M., Chen, D., Bahler, J., Jones, N., Morgan, B.A., and Quinn, J. (2002). The *Srk1* protein kinase is a target for the *Sty1* stress-activated MAPK in fission yeast. *J Biol Chem* 277, 33411-33421.
- Solsbacher, J., Maurer, P., Bischoff, F.R., and Schlenstedt, G. (1998). Cse1p is involved in export of yeast importin alpha from the nucleus. *Mol Cell Biol* 18, 6805-6815.
- Song, J., Durrin, L.K., Wilkinson, T.A., Krontiris, T.G., and Chen, Y. (2004). Identification of a SUMO-binding motif that recognizes SUMO-modified proteins. *PNAS* 101, 14373-14378.
- Song, L., and Rape, M. (2008). Reverse the curse--the role of deubiquitination in cell cycle control. *Curr Opin Cell Biol* 20, 156-163.
- Soucy, T.A., Smith, P.G., and Rolfe, M. (2009). Targeting NEDD8-activated cullin-RING ligases for the treatment of cancer. *Clin Cancer Res* 15, 3912-3916.
- Staniek, K., and Nohl, H. (2000). Are mitochondria a permanent source of reactive oxygen species? *Biochimica et Biophysica Acta* 1460, 268-275.
- Steffan, J.S., Agrawal, N., Pallos, J., Rockabrand, E., Trotman, L.C., Slepko, N., Illes, K., Lukacovich, T., Zhu, Y.Z., Cattaneo, E., *et al.* (2004). SUMO modification of Huntingtin and Huntington's disease pathology. *Science* 304, 100-104.
- Stegmeier, F., Rape, M., Draviam, V.M., Nalepa, G., Sowa, M.E., Ang, X.L., McDonald, E.R., 3rd, Li, M.Z., Hannon, G.J., Sorger, P.K., *et al.* (2007). Anaphase initiation is regulated by antagonistic ubiquitination and deubiquitination activities. *Nature* 446, 876-881.
- Stephan, J.S., Yeh, Y.Y., Ramachandran, V., Deminoff, S.J., and Herman, P.K. (2009). The Tor and PKA signaling pathways independently target the Atg1/Atg13 protein kinase complex to control autophagy. *PNAS* 106, 17049-17054.
- Storz, G., Tartaglia, L.A., and Ames, B.N. (1990). Transcriptional regulator of oxidative stress-inducible genes: direct activation by oxidation. *Science* 248, 189-194.

- Su, S.S., Tanaka, Y., Samejima, I., Tanaka, K., and Yanagida, M. (1996). A nitrogen starvation-induced dormant G0 state in fission yeast: the establishment from uncommitted G1 state and its delay for return to proliferation. *J Cell Sci* 109 ( Pt 6), 1347-1357.
- Sugawara, K., Suzuki, N.N., Fujioka, Y., Mizushima, N., Ohsumi, Y., and Inagaki, F. (2004). The crystal structure of microtubule-associated protein light chain 3, a mammalian homologue of *Saccharomyces cerevisiae* Atg8. *Genes To Cells* 9, 611-618.
- Sugiura, R., Toda, T., Dhut, S., Shuntoh, H., and Kuno, T. (1999). The MAPK kinase Pek1 acts as a phosphorylation-dependent molecular switch. *Nature* 399, 479-483.
- Sun, X.Z., Vinci, C., Makmura, L., Han, S., Tran, D., Nguyen, J., Hamann, M., Grazziani, S., Sheppard, S., Gutova, M., *et al.* (2003). Formation of disulfide bond in p53 correlates with inhibition of DNA binding and tetramerization. *Antioxidants & Redox Signaling* 5, 655-665.
- Sun, Z.W., and Allis, C.D. (2002). Ubiquitination of histone H2B regulates H3 methylation and gene silencing in yeast. *Nature* 418, 104-108.
- Sundaresan, M., Yu, Z.X., Ferrans, V.J., Irani, K., and Finkel, T. (1995). Requirement for generation of H<sub>2</sub>O<sub>2</sub> for platelet-derived growth factor signal transduction. *Science* 270, 296-299.
- Suzuki, K., Kirisako, T., Kamada, Y., Mizushima, N., Noda, T., and Ohsumi, Y. (2001). The pre-autophagosomal structure organized by concerted functions of APG genes is essential for autophagosome formation. *EMBO* 20, 5971-5981.
- Suzuki, N.N., Yoshimoto, K., Fujioka, Y., Ohsumi, Y., and Inagaki, F. (2005). The crystal structure of plant ATG12 and its biological implication in autophagy. *Autophagy* 1, 119-126.
- Svejstrup, J.Q. (2007). Elongator complex: how many roles does it play? *Curr Opin Cell Biol* 19, 331-336.
- Swords, R.T., Kelly, K.R., Smith, P.G., Garnsey, J.J., Mahalingam, D., Medina, E., Oberheu, K., Padmanabhan, S., O'Dwyer, M., Nawrocki, S.T., *et al.* (2010). Inhibition of NEDD8-activating enzyme: a novel approach for the treatment of acute myeloid leukemia. *Blood* 115, 3796-3800.
- Taherbhoy, A.M., Tait, S.W., Kaiser, S.E., Williams, A.H., Deng, A., Nourse, A., Hammel, M., Kurinov, I., Rock, C.O., Green, D.R., *et al.* (2011). Atg8 transfer from Atg7 to Atg3: a distinctive E1-E2 architecture and mechanism in the autophagy pathway. *Mol Cell* 44, 451-461.



Takada, H., Nishimura, M., Asayama, Y., Mannse, Y., Ishiwata, S., Kita, A., Doi, A., Nishida, A., Kai, N., Moriuchi, S., *et al.* (2007). Atf1 is a target of the mitogen-activated protein kinase Pmk1 and regulates cell integrity in fission yeast. *Mol Biol Cell* 18, 4794-4802.

Takeda, K., and Ichijo, H. (2002). Neuronal p38 MAPK signalling: an emerging regulator of cell fate and function in the nervous system. *Genes To Cells* 7, 1099-1111.

Takeda, K., Yoshida, T., Kikuchi, S., Nagao, K., Kokubu, A., Pluskal, T., Villar-Briones, A., Nakamura, T., and Yanagida, M. (2010). Synergistic roles of the proteasome and autophagy for mitochondrial maintenance and chronological lifespan in fission yeast. *PNAS* 107, 3540-3545.

Takeda, T., Toda, T., Kominami, K., Kohnosu, A., Yanagida, M., and Jones, N. (1995). *Schizosaccharomyces pombe atf1<sup>+</sup>* encodes a transcription factor required for sexual development and entry into stationary phase. *EMBO* 14, 6193-6208.

Tal, R., Winter, G., Ecker, N., Klionsky, D.J., and Abeliovich, H. (2007). Aup1p, a yeast mitochondrial protein phosphatase homolog, is required for efficient stationary phase mitophagy and cell survival. *J Biol Chem* 282, 5617-5624.

Tanida, I., Mizushima, N., Kiyooka, M., Ohsumi, M., Ueno, T., Ohsumi, Y., and Kominami, E. (1999). Apg7p/Cvt2p: A novel protein-activating enzyme essential for autophagy. *Mol Biol Cell* 10, 1367-1379.

Tanida, I., Tanida-Miyake, E., Komatsu, M., Ueno, T., and Kominami, E. (2002). Human Apg3p/Aut1p homologue is an authentic E2 enzyme for multiple substrates, GATE-16, GABARAP, and MAP-LC3, and facilitates the conjugation of hApg12p to hApg5p. *J Biol Chem* 277, 13739-13744.

Tateishi, K., Omata, M., Tanaka, K., and Chiba, T. (2001). The NEDD8 system is essential for cell cycle progression and morphogenetic pathway in mice. *J Cell Biol* 155, 571-579.

Tobiume, K., Matsuzawa, A., Takahashi, T., Nishitoh, H., Morita, K., Takeda, K., Minowa, O., Miyazono, K., Noda, T., and Ichijo, H. (2001). ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. *EMBO reports* 2, 222-228.

Toda, T., Dhut, S., Superti-Furga, G., Gotoh, Y., Nishida, E., Sugiura, R., and Kuno, T. (1996). The fission yeast pmk1<sup>+</sup> gene encodes a novel mitogen-activated protein kinase homolog which regulates cell integrity and functions coordinately with the protein kinase C pathway. *Mol Cell Biol* 16, 6752-6764.

- Toda, T., Shimanuki, M., and Yanagida, M. (1991). Fission yeast genes that confer resistance to staurosporine encode an AP-1-like transcription factor and a protein kinase related to the mammalian ERK1/MAP2 and budding yeast FUS3 and KSS1 kinases. *Gene Dev* 5, 60-73.
- Tokgoz, Z., Siepmann, T.J., Streich, F., Jr., Kumar, B., Klein, J.M., and Haas, A.L. (2012). E1-E2 interactions in ubiquitin and Nedd8 ligation pathways. *J Biol Chem* 287, 311-321.
- Tolbert, B.S., Tajc, S.G., Webb, H., Snyder, J., Nielsen, J.E., Miller, B.L., and Basavappa, R. (2005). The active site cysteine of ubiquitin-conjugating enzymes has a significantly elevated pKa: functional implications. *Biochemistry* 44, 16385-16391.
- Toone, W.M., Kuge, S., Samuels, M., Morgan, B.A., Toda, T., and Jones, N. (1998). Regulation of the fission yeast transcription factor Pap1 by oxidative stress: requirement for the nuclear export factor Crm1 (Exportin) and the stress-activated MAP kinase Sty1/Spc1. *Gene Dev* 12, 1453-1463.
- Tsukada, M., and Ohsumi, Y. (1993). Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae*. *FEBS letters* 333, 169-174.
- Turrens, J.F. (1997). Superoxide production by the mitochondrial respiratory chain. *Bioscience Reports* 17, 3-8.
- Umeda, M., Izaddoost, S., Cushman, I., Moore, M.S., and Sazer, S. (2005). The fission yeast *Schizosaccharomyces pombe* has two importin-alpha proteins, Imp1p and Cut15p, which have common and unique functions in nucleocytoplasmic transport and cell cycle progression. *Genetics* 171, 7-21.
- Ushio-Fukai, M. (2006). Redox signaling in angiogenesis: role of NADPH oxidase. *Cardiovascular Res* 71, 226-235.
- Van der Veen, A.G., Schorpp, K., Schlieker, C., Buti, L., Damon, J.R., Spooner, E., Ploegh, H.L., and Jentsch, S. (2011). Role of the ubiquitin-like protein Urm1 as a noncanonical lysine-directed protein modifier. *PNAS* 108, 1763-1770.
- van Wijk, S.J., and Timmers, H.T. (2010). The family of ubiquitin-conjugating enzymes (E2s): deciding between life and death of proteins. *FASEB J* 24, 981-993.
- Vandenbroucke, K., Robbens, S., Vandepoele, K., Inze, D., Van de Peer, Y., and Van Breusegem, F. (2008). Hydrogen peroxide-induced gene expression across kingdoms: a comparative analysis. *Mol Biol Evol* 25, 507-516.
- Veal, E.A., Day, A.M., and Morgan, B.A. (2007). Hydrogen peroxide sensing and signaling. *Mol Cell* 26, 1-14.

Veal, E.A., Findlay, V.J., Day, A.M., Bozonet, S.M., Evans, J.M., Quinn, J., and Morgan, B.A. (2004). A 2-Cys peroxiredoxin regulates peroxide-induced oxidation and activation of a stress-activated MAP kinase. *Mol Cell* 15, 129-139.

Velu, C.S., Niture, S.K., Doneanu, C.E., Pattabiraman, N., and Srivenugopal, K.S. (2007). Human p53 is inhibited by glutathionylation of cysteines present in the proximal DNA-binding domain during oxidative stress. *Biochemistry* 46, 7765-7780.

Verma, R., Feldman, R.M., and Deshaies, R.J. (1997). SIC1 is ubiquitinated in vitro by a pathway that requires CDC4, CDC34, and cyclin/CDK activities. *Mol Biol Cell* 8, 1427-1437.

Vivancos, A.P., Castillo, E.A., Biteau, B., Nicot, C., Ayte, J., Toledano, M.B., and Hidalgo, E. (2005). A cysteine-sulfinic acid in peroxiredoxin regulates H<sub>2</sub>O<sub>2</sub>-sensing by the antioxidant Pap1 pathway. *PNAS* 102, 8875-8880.

Vivancos, A.P., Castillo, E.A., Jones, N., Ayte, J., and Hidalgo, E. (2004). Activation of the redox sensor Pap1 by hydrogen peroxide requires modulation of the intracellular oxidant concentration. *Mol Microbiol* 52, 1427-1435.

Vivancos, A.P., Jara, M., Zuin, A., Sanso, M., and Hidalgo, E. (2006). Oxidative stress in *Schizosaccharomyces pombe*: different H<sub>2</sub>O<sub>2</sub> levels, different response pathways. *MGG* 276, 495-502.

Walden, H., Podgorski, M.S., and Schulman, B.A. (2003). Insights into the ubiquitin transfer cascade from the structure of the activating enzyme for NEDD8. *Nature* 422, 330-334.

Wang, F., Liu, M., Qiu, R., and Ji, C. (2011). The dual role of ubiquitin-like protein Urm1 as a protein modifier and sulfur carrier. *Protein & Cell* 2, 612-619.

Wang, Y., Gulis, G., Buckner, S., Johnson, P.C., Sullivan, D., Busenlehner, L., and Marcus, S. (2010). The MAP kinase Pmk1 and protein kinase A are required for rotenone resistance in the fission yeast, *Schizosaccharomyces pombe*. *Biochem Biophys Res Comm* 399, 123-128.

Waskiewicz, A.J., and Cooper, J.A. (1995). Mitogen and stress response pathways: MAP kinase cascades and phosphatase regulation in mammals and yeast. *Curr Opin Cell Biol* 7, 798-805.

Watanabe, Y., Lino, Y., Furuhashi, K., Shimoda, C., and Yamamoto, M. (1988). The *S.pombe mei2* gene encoding a crucial molecule for commitment to meiosis is under the regulation of cAMP. *EMBO* 7, 761-767.

- Watanabe, Y., and Yamamoto, M. (1996). *Schizosaccharomyces pombe pcr1<sup>+</sup>* encodes a CREB/ATF protein involved in regulation of gene expression for sexual development. *Mol Cell Biol* 16, 704-711.
- Wei, Y., Pattingre, S., Sinha, S., Bassik, M., and Levine, B. (2008). JNK1-mediated phosphorylation of Bcl-2 regulates starvation-induced autophagy. *Mol Cell* 30, 678-688.
- Welchman, R.L., Gordon, C., and Mayer, R.J. (2005). Ubiquitin and ubiquitin-like proteins as multifunctional signals. *Nat Rev Mol Cell Biol* 6, 599-609.
- Wilkinson, M.G., Samuels, M., Takeda, T., Toone, W.M., Shieh, J.C., Toda, T., Millar, J.B., and Jones, N. (1996). The Atf1 transcription factor is a target for the Sty1 stress-activated MAP kinase pathway in fission yeast. *Gene Dev* 10, 2289-2301.
- Wohlschlegel, J.A., Johnson, E.S., Reed, S.I., and Yates, J.R., 3rd (2004). Global analysis of protein sumoylation in *Saccharomyces cerevisiae*. *J Biol Chem* 279, 45662-45668.
- Woo, H.A., Yim, S.H., Shin, D.H., Kang, D., Yu, D.Y., and Rhee, S.G. (2010). Inactivation of peroxiredoxin I by phosphorylation allows localized H<sub>2</sub>O<sub>2</sub> accumulation for cell signaling. *Cell* 140, 517-528.
- Wood, Z.A., Poole, L.B., and Karplus, P.A. (2003a). Peroxiredoxin evolution and the regulation of hydrogen peroxide signaling. *Science* 300, 650-653.
- Wood, Z.A., Schroder, E., Robin Harris, J., and Poole, L.B. (2003b). Structure, mechanism and regulation of peroxiredoxins. *Trends Biochem Sci* 28, 32-40.
- Wu, J.J., Quijano, C., Chen, E., Liu, H., Cao, L., Fergusson, M.M., Rovira, II, Gutkind, S., Daniels, M.P., Komatsu, M., *et al.* (2009). Mitochondrial dysfunction and oxidative stress mediate the physiological impairment induced by the disruption of autophagy. *Aging* 1, 425-437.
- Wu, K., Yamoah, K., Dolios, G., Gan-Erdene, T., Tan, P., Chen, A., Lee, C.G., Wei, N., Wilkinson, K.D., Wang, R., *et al.* (2003a). DEN1 is a dual function protease capable of processing the C terminus of Nedd8 and deconjugating hyper-neddylated CUL1. *J Biol Chem* 278, 28882-28891.
- Wu, P.Y., Hanlon, M., Eddins, M., Tsui, C., Rogers, R.S., Jensen, J.P., Matunis, M.J., Weissman, A.M., Wolberger, C., and Pickart, C.M. (2003b). A conserved catalytic residue in the ubiquitin-conjugating enzyme family. *EMBO* 22, 5241-5250.
- Xi, J., Ge, Y., Kinsland, C., McLafferty, F.W., and Begley, T.P. (2001). Biosynthesis of the thiazole moiety of thiamin in *Escherichia coli*: identification of an acyldisulfide-

linked protein--protein conjugate that is functionally analogous to the ubiquitin/E1 complex. *PNAS* 98, 8513-8518.

Xie, Z., and Klionsky, D.J. (2007). Autophagosome formation: core machinery and adaptations. *Nat Cell Biol* 9, 1102-1109.

Xie, Z., Nair, U., and Klionsky, D.J. (2008). Atg8 controls phagophore expansion during autophagosome formation. *Mol Biol Cell* 19, 3290-3298.

Xirodimas, D.P. (2008). Novel substrates and functions for the ubiquitin-like molecule NEDD8. *Biochemical Society Transactions* 36, 802-806.

Xirodimas, D.P., Saville, M.K., Bourdon, J.C., Hay, R.T., and Lane, D.P. (2004). Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. *Cell* 118, 83-97.

Xu, J., Zhang, J., Wang, L., Zhou, J., Huang, H., Wu, J., Zhong, Y., and Shi, Y. (2006). Solution structure of Urm1 and its implications for the origin of protein modifiers. *PNAS* 103, 11625-11630.

Xu, Z., Lam, L.S., Lam, L.H., Chau, S.F., Ng, T.B., and Au, S.W. (2008). Molecular basis of the redox regulation of SUMO proteases: a protective mechanism of intermolecular disulfide linkage against irreversible sulfhydryl oxidation. *FASEB J* 22, 127-137.

Yamada, K., Nakagawa, C.W., and Mutoh, N. (1999). *Schizosaccharomyces pombe* homologue of glutathione peroxidase, which does not contain selenocysteine, is induced by several stresses and works as an antioxidant. *Yeast* 15, 1125-1132.

Yamada, Y., Suzuki, N.N., Hanada, T., Ichimura, Y., Kumeta, H., Fujioka, Y., Ohsumi, Y., and Inagaki, F. (2007). The crystal structure of Atg3, an autophagy-related ubiquitin carrier protein (E2) enzyme that mediates Atg8 lipidation. *J Biol Chem* 282, 8036-8043.

Yarian, C., Townsend, H., Czystkowski, W., Sochacka, E., Malkiewicz, A.J., Guenther, R., Miskiewicz, A., and Agris, P.F. (2002). Accurate translation of the genetic code depends on tRNA modified nucleosides. *J Biol Chem* 277, 16391-16395.

Yorimitsu, T., and Klionsky, D.J. (2005). Autophagy: molecular machinery for self-eating. *Cell Death Diff* 12:2, 1542-1552.

Yu, L., Wan, F., Dutta, S., Welsh, S., Liu, Z., Freundt, E., Baehrecke, E.H., and Lenardo, M. (2006). Autophagic programmed cell death by selective catalase degradation. *PNAS* 103, 4952-4957.

Zaitsevskaya-Carter, T., and Cooper, J.A. (1997). Spm1, a stress-activated MAP kinase that regulates morphogenesis in *S.pombe*. *EMBO* 16, 1318-1331.

- Zhang, H., Bosch-Marce, M., Shimoda, L.A., Tan, Y.S., Baek, J.H., Wesley, J.B., Gonzalez, F.J., and Semenza, G.L. (2008). Mitochondrial autophagy is an HIF-1-dependent adaptive metabolic response to hypoxia. *J Biol Chem* 283, 10892-10903.
- Zhao, J. (2007). Sumoylation regulates diverse biological processes. *CMLS* 64, 3017-3033.
- Zheng, M., Aslund, F., and Storz, G. (1998). Activation of the OxyR transcription factor by reversible disulfide bond formation. *Science* 279, 1718-1721.
- Zheng, N., Wang, P., Jeffrey, P.D., and Pavletich, N.P. (2000). Structure of a c-Cbl-UbcH7 complex: RING domain function in ubiquitin-protein ligases. *Cell* 102, 533-539.
- Zhou, W., Ryan, J.J., and Zhou, H. (2004). Global analyses of sumoylated proteins in *Saccharomyces cerevisiae*. Induction of protein sumoylation by cellular stresses. *J Biol Chem* 279, 32262-32268.
- Zhou, X., Ma, Y., Sugiura, R., Kobayashi, D., Suzuki, M., Deng, L., and Kuno, T. (2010). MAP kinase kinase kinase (MAPKKK)-dependent and -independent activation of Sty1 stress MAPK in fission yeast. *J Biol Chem* 285, 32818-32823.
- Zuin, A., Carmona, M., Morales-Ivorra, I., Gabrielli, N., Vivancos, A.P., Ayte, J., and Hidalgo, E. (2010). Lifespan extension by calorie restriction relies on the Sty1 MAP kinase stress pathway. *EMBO* 29, 981-991.
- Zuin, A., Gabrielli, N., Calvo, I.A., Garcia-Santamarina, S., Hoe, K.L., Kim, D.U., Park, H.O., Hayles, J., Ayte, J., and Hidalgo, E. (2008). Mitochondrial dysfunction increases oxidative stress and decreases chronological life span in fission yeast. *PloS one* 3, e2842.

## **Appendix**